IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Arlene RAMSINGH *et al.*

Serial No.: 09/879,572

Filing Date: June 12, 2001

For: COXSACKIEVIRUS B4 EXPRESSION VECTORS AND USES THEREOF

Examiner: Stacy Chen

Group Art Unit: 1648

Atty Dkt: 29025.0001

Customer No.

26694

PATENT TRADEMARK OFFICE

**DECLARATION OF STEVEN TRACY, Ph.D., PURSUANT TO 37 C.F.R § 1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am an expert in the field of viral biology and molecular virology and have worked with coxsackieviruses and other Picornaviruses for 25 years. I am an author/co-author of about 85 peer-reviewed publications, review articles and book chapters in this field. My *Curriculum Vitae* is attached. I am currently a Professor of Pathology & Microbiology at the University of Nebraska Medical Center. I am very familiar with the research publications of Dr. Arlene Ramsingh, co-inventor of the above-identified patent application. We are colleagues in the same field.

2. I have reviewed the relevant sections of the outstanding Office Action and the patent application, including the rejected claims. My comments below are addressed to several points raised by the Examiner, particularly in relation to her citation of the reference Caggana, M, Chan, P, and Ramsingh, A (1993) *J. Virol.* 67:4797-4803 (referred to below as "Caggana") as anticipating claims 1, 3, 4, 18, and 20-26.

3. I wish to preface my specific remarks with the following general statements. Simply put, virus populations, especially RNA virus populations in which no editing function exists in the RNA-dependent RNA polymerase, are constantly changing. The extent to which change occurs is a function of how the virus population at present adapts to the current environment. Merely inoculating cells with a dose of an RNA virus results in a new population of virus in which changes in the RNA can be found which were not present prior to the passage. Virus populations are not cast in concrete, but are plastic. Such differences do not necessarily make non-identical viruses "heterologous" to one another. It is important to keep this in mind when trying to "equate" notions of homology and heterology in viruses, especially RNA viruses, with the similar concepts applied to DNA whether from *E. coli*, yeast or mammalian cells. It is hoped that my remarks will help clarify these issues.

4. As regards viruses of the CB4 serotype, JVB can be considered a strain of the CB4 *serotype*; CB4-P and CB4-V can be considered either variants of that strain, variants of one another or different strains. That distinction is not really helpful. Because CB4-V was derived from CB4-P by mouse passage, the CB4-V strain or variant is clearly very closely related "structurally" to the CB4-P strain or variant, sharing near-perfect identity. They may be considered "homologues" of one another; they certainly are not heterologous. So even though they lack 100% sequence identity, CB4-V and CB4-P cannot be viewed as heterologous to one another in terms of viral biology. It is well known in the picornavirus field that genetic variations *within* a particular group can have diverse biological or "clinical" phenotypes. For example, at certain doses, avirulent or non-pathogenic genetic variants of the JVB strain of CB4, like CB4-P, cause mild, transient pancreatitis; other more virulent mutants, like CB4-V may cause chronic pancreatitis or transient diabetes in mice. Again, this is not a basis for asserting that they are heterologous.

5. The Examiner has made the following statements:

(a) Caggana teaches coxsackievirus CB4-P/CB4-V chimeras, in which an attenuated strain, CB4-P expresses heterologous CB4-V proteins of various types (P1, P2, P3) at various regions of the CB4-P genome, including just downstream from codon 129 of VP1, DE loop (see page 4797- 4798, "Construction of recombinant viruses"; page 4798, Figure 1; pages 4799-4801, bridging paragraph; and page 4802, second column, first line).

(b) The VP1 region encodes capsid, which itself is immunogenic and thus contains epitopes (B-cell and/or T-cell).

(c) Applicant has argued that Caggana's chimerics are not intended to be encompassed by the instant claims because Caggana replaces regions of CB4 viruses with other regions of CB4 viruses. Applicant argues that the replacement of CB4-P genes with CB4-V genes is not a heterologous nucleic acid insertion. While the Office acknowledges that the CB4-P and CB4-V strains of coxsackievirus differ by about five amino acids, they remain structurally distinct strains because they have different amino acid sequences that renders one virulent and the other non-virulent. Even though the virulence is credited to one amino acid residue in the capsid protein of VP1 (Caggana, abstract), the sequences of the two remain different. The VP1 region of CB4-P is not *the same* as the VP1 region of CB4-V structurally (amino acid difference) and functionally (virulent, non-virulent). As such, Caggana's chimeric meets the claim limitations of being a CB4 virion with heterologous nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion, wherein heterologous nucleic acid is defined as "not otherwise naturally present in the genome of the virus". In the instant case, the P1 region of CB4-V was not naturally present in the genome of the CB4-P virus. Therefore, the claims are encompassed by Caggana.

6. The chimeras (=chimerae) to which the Examiner refers (at Para. 5(a), line 1, in the above quote) are merely man-made variants of either strain, any of which might occur given time and correct circumstances; the use of recombinant DNA technology permits immediate analysis instead. Technically speaking, one might refer to such minor changes as intratypic chimeric viruses, to denote the fact that one **homologous** region of a related strain of the same serotype was used to **replace** the original sequence. Thus, whether one mutates a nucleotide to change one amino acid, or one excises the coding sequence for 20 contiguous amino acids and puts in its place the mutated coding sequence in which the same nucleotide has been mutated, the outcome is the same. Viruses of the present claims **are also chimeras** -- characterized in this case by the *insertion* of truly heterologous sequences such as ovalbumin or HIV peptides. The process of cloning described by Caggana involved removal of a CB4 sequence, and re-insertion of another related and closely homologous (which is to say, few differences at the nucleotide level) CB4 sequence into that space - a 'functional sequence replacement'. As the re-inserted similar sequence was from a very closely related CB4 genome with extremely few differences at the nucleotide and amino acid levels, this represents *replacement* of one sequence with another, nearly identical sequence. It is not insertion, as would be the case if the coding sequence for a non-CB protein such as green fluorescent protein (or an HIV peptide) were inserted into the intact CB genome, making such chimeric virus's nucleic acid molecule longer than that of the parental virus.

7. Again, the CB4 nucleic acid sequence (from the -V or -P variant) that replaces the original CB4 sequence in the other virus should not be considered heterologous, *as long as* the replacing sequence is the former sequence's ***homologue*** in the donor genome. The Examiner's extreme interpretation of the definition of "heterologous" used in the application

> "[t]he term 'heterologous polypeptide' refers to a polypeptide which is not otherwise naturally expressed by the virus. The term 'heterologous nucleic acid' refers to any nucleic acid which is not otherwise naturally present in the genome of the virus at the position in which it is inserted"

makes poor biological sense, in my opinion. This is indeed the case in Caggana when replacing the CB4-P protein 1D with the protein 1D from CB4-V). While the Office is correct in stating that "[t]he VP1 region of CB4-P is not *the same* as the VP1 region of CB4-V structurally (amino acid difference) and functionally (virulent, non-virulent)," I disagree with the notion that Caggana's "chimeric" (see my comment above) is "a CB4 virion with **heterologous** nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion." It seems to be a contradiction in terms to view a homologous gene, sequence, or protein as "heterologous." In the case of the viruses described in Caggana, it is clear to me that the replacement sequence is **not heterologous.**

8. To summarize, the cloning strategy described in the present application to create the claimed recombinant viruses and nucleic acid molecules differs fundamentally from that described by Caggana in that, here, truly heterologous sequences (such as ovalbumin, HIV gag p24, proteins that are not found in CB viruses **in nature**[1]), that include additional coding sequence are inserted into CB4 cloning vectors (not merely replacing existing, homologous sequences). Because this is "insertional cloning," the overall coding capacity of the recombinant virus increases significantly compared to that of the "host" CB4 strain's genome. The viruses and the manipulations described in Caggana are distinct from the claimed recombinant chimeric viruses and the methods used to generate them, as described in this application.

1 or, as stated in the words of the Application's definition of "heterologous": "a polypeptide which is not ... ***naturally*** expressed by the virus..." or "any nucleic acid which is not ... ***naturally*** present in the genome of the virus..." *(emphasis added)*

9. Viruses that are genetically *equivalent* to CB4-P can be manipulated genetically per the application to allow insertions of heterologous nucleic acids in defined spots as described, *e.g.*, to yield an internal fusion of VP1 (see claim 7) or inserted in-frame and directly upstream of VP4 coding sequences (see claim 13). I know of no reason why one could not use, therefore, any strain of CB4 (or any of the 6 CB serotypes), to produce the chimerae described by the Applicants. There is no reason that a skilled person would be limited in his/her ability to practice this invention using CB4-P or any other CB4 virus. Any genetic variant of the JVB strain of the CB4 serotype can be used in the very same way. JVB is publicly available from the ATCC. Thus, the skilled person would not have to resort to any other deposits of CB4-P virus to practice this invention fully. Moreover, there is nothing special about the serotype B4 versus the other five CB serotypes. Once a person skilled in the field has been apprised of the present invention and read the application, that person will be able to practice it as written in the claims. Indeed, what works in CB4-P to create a virus expressing a heterologous polypeptide that can act a as an immunogen would also work in CB4-JVB or CB4-V (notwithstanding other considerations like virulence). Indeed, what works in CB4-P would also work in any strain of virus of the other coxsackievirus B serotypes.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

5-22-05

Date

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| 1968-1972 | B.A., With Honors<br>Major Field: Biology<br>University of California, San Diego (UCSD), La Jolla, CA |
| 1972-1979 | Ph.D.<br>Department of Biology, UCSD<br>La Jolla, CA<br>Thesis Advisors: David E. Kohne, Ph.D. and William C. Baxt, M.D. |

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| Jul 1990-Jun 1997 | Associate Professor, Department of Pathology and Microbiology, COM, UNMC, Omaha NE |
| Feb 1987-Jun 1990 | Assistant Professor, Department of Pathology and Microbiology, COM, UNMC, Omaha NE |
| Jun 1985-Feb 1987 | Chief, Molecular Virology Group (*Arbeitsgruppe Molekulare Virologie*),<br>German Primate Center (*Deutsches Primatenzentrum*), Göttingen, Germany |
| Mar 1985-Jun 1985 | Visiting Assistant Professor, Department of Microbiology, Health Science Center,<br>University of Texas, San Antonio, TX |
| Nov 1981-Jun 1985 | Assistant Research Microbiologist, School of Public Health<br>University of California, Berkeley, CA |
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PATENT TRADEMARK OFFICE

**DECLARATION OF BARBARA WEISER, M.D. PURSUANT TO 37 C.F.R § 1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am an expert in the field of molecular virology and clinical infectious disease, with an emphasis on HIV. I have worked with various different classes of viruses for about 25 years and have focused my research on the RNA virus HIV-1 for the past 21 years. I am an author/co-author of about 60 peer-reviewed publications, review articles and book chapters in this field. My *Curriculum Vitae* is attached. I am currently a Co-Director of HIV Research at the Wadsworth Center, New York State Department of Health, and a Professor of Medicine, Division of HIV Medicine, Albany Medical College. I have been an active member of the NIH Women's Interagency HIV Study (*WIHS*) Laboratory Committee since 1993 and have chaired the subcommittees on Virology, and HIV Infection in Drug-Using Women. I am currently a member of the *WIHS* Scientific Advisory Board and chair of its Scientific Working Group on HIV Virology. I am familiar with the research of Dr. Arlene Ramsingh, co-inventor of the above-identified patent application (particularly as it concerns potential HIV immunogens) as we been staff members in the same institution for the past 14 years.

2. I have reviewed the relevant sections of the Office Action and the patent application, including the claims. My comments below are based on my specialized knowledge of RNA viruses and their particular properties that distinguish them from DNA viruses and from prokaryotic and eukaryotic cells as concerns their nucleic acids, replication and unique structural variation and heterogeneity. Although I am not a specific expert in coxsackieviruses, I do know a great deal about how they and other RNA viruses resemble and also differ in significant ways from HIV-1. I note that the present invention does focus to a large degree on production and use of recombinant CB4 viruses into which have been inserted heterologous nucleotide sequences that encode HIV peptides for use as immunogens and vaccines.

3. My research focuses on HIV-1 sequence variation and its impact on pathogenesis and treatment of HIV and AIDS. HIV-1 variation is ubiquitous and occurs not only among infected individuals, but also among HIV-1 strains or "quasispecies" obtained from different organs in the same person. HIV-1 envelope genes isolated from people in Africa and New York are highly divergent and may differ by ~25-30% of their sequences. HIV-1 and coxsackie genomes diverge from one another by much more than that, of course. It is highly probable that the diversity of HIV-1 sequences among virions within the body of a single patient across various compartments (blood, lymph, lymphatic tissue, mucosal tissue of the gut and reproductive system, *etc.*) is greater than the sum total of diversity of all CB4 viruses throughout the world. That fact has been proven to be true when comparing HIV-1 diversity in one patient with the influenza virus worldwide. Having said that, it is important to appreciate for the present discussion that the genes and their polypeptide products in all these HIV-1 variants (some differing quite extensively, *e.g.*, by 25-30% of the total genome) are nonetheless **not considered to be "heterologous"** one to the other.

4. In view of the above, I was rather stunned to learn that the Patent Office, when considering the publication by Caggana *et al. J Virol.* (1993) *67*:4797-803) and applying it to the invention being claimed in the above-identified application, took the position that the CB4-P and CB4-V variants were heterologous to one another. That is a view that would simply not be accepted by any virologist (or other biologist) for reasons presented below. In comparing these two variants over a stretch of close to 3300 nt's (including the most variable part of the viral genome), only 9

total nucleotides differed, of which only 5 were in coding regions, resulting in viruses that differed in a grand total of 5 amino acids in three viral proteins. A single amino acid position in the VP1 protein was shown to be responsible for the phenotype of interest (virulence in mice). This amount of variation is exceedingly small and is nowhere near the extent that would be considered as defining separate viral serotypes or strains or classes or whatever other taxonomic term one chooses, and would certainly not be considered by people skilled in this field as "heterologous". Indeed, as I relate below in Sec. 7, major "functional" differences in HIV-1 pathogenicity that are associated with even greater nucleotide sequence differences than those which distinguish CB4-V from CB4-P, are not considered heterologous by anyone's definition of the term.

5. Another reason why the "new" CB4 nucleic acid sequence (CB4-V), described in the Caggana paper and replacing the original CB4 sequence in the CB4-P variant, cannot fairly be considered heterologous is because these sequences are, in fact, **homologous:** they encode the same protein with the same biological function, but with one mutated amino acid. Something that is, in fact, homologous cannot at the same time be heterologous. That situation is completely distinct from this invention - where sequences truly foreign to CB4 viruses are inserted in certain sites to be appropriately expressed so that these recombinant virions can serve as immunogens to evoke immune responses to these non-coxsackievirus peptides.6. The Examiner has focused on this minimal CB4-V - CB4-P difference in conjunction with the definition for "heterologous" used in the application and asserted first that:

> Even though the virulence is credited to one amino acid residue in the capsid protein of VP1 ..., the sequences of the two remain different. The VP1 region of CB4-P is not ***the same as*** the VP1 region of CB4-V structurally (amino acid difference) and functionally (virulent, non-virulent).

(emphasis added). Those statements are accurate and correct. From this, the Examiner went on to characterize the virus described in the Caggana paper as having

> ...**heterologous** nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion, wherein heterologous nucleic acid is defined as "not otherwise naturally present in the genome of the virus". ... the P1 region of CB4-V was not naturally present in the genome of the CB4-P virus.

The Examiner's interpretation of the definition of "heterologous" used in the application is strained, and not in line with how those in the field view RNA viruses, their nucleic acids, and their proteins, the variation in the sequences of these molecules, and their relationship with one another. It is these relationships that are at the heart of the concepts of 'homologous' and "heterologous' (because something is homologous or heterologous only to a reference sequence or virus or bacterium or animal species). Thus, I must firmly disagree with the Patent Office's assertion that the virus described in Caggana is

> "a CB4 virion with heterologous nucleic acid inserted into an open reading frame that results in fusion to the capsid protein of the virion".

Rather, it is a CB4 virus in which a segment of a nucleic acid (a viral gene) has been replaced with the **homologous** (albeit non-identical) segment from a very closely related virus of the same taxon. Calling it heterologous is the polar opposite of what I and scientists skilled in this field would call it, the application's definition notwithstanding. Reading the application alone, it is unequivocal to me that the Applicants did not intend to include within their definition of "heterologous polypeptides" other coxsackievirus polypeptides, and certainly not *homologous* CB4 polypeptides as in Caggana.

7. It may be instructive to compare the situation of the CB4-P and CB4-V viruses of Caggana with differences between HIV-1 viruses where defined sequence differences are associated with different levels of pathogenicity. In studying determinants of HIV-1 attenuation and pathogenesis, we and others have turned our attention to "long term non-progressors" (LTNP), patients infected with the virus for say ten or more year but in whom (a) the disease does not progress and (b) there is no significant depletion of CD4+ T cells. We reported on an individual[1] who underwent a transition from LTNP to rapidly progressive infection and analyzed hundreds of clones of full-length HIV-1 RNA genomes from the plasma of this individual before and during the transition. We found that a 20 bp deletion appeared to confer attenuation on the virus. When the infection became progressive, all viruses had intact (non-deleted) sequences and were derived from

[1] Fang G, Burger H, Chappey C, Rowland-Jones S, Visosky A, Chen C, Moran T, Townsend L, Murray M, and Weiser B. (2001) Analysis of transition from long-term non-progressive to progressive infection identifies sequences which may attenuate HIV-1, *AIDS Res and Hum Retroviruses*, 17:1395-1404

a minor species present earlier. Comparing this to the CB4-V and CB4-P viruses described in Caggana, one sees that a defined molecular change, somewhat larger in magnitude in our case, was responsible for the virus's pathogenicity. However, the sequence with the 20 bp deletion was not "heterologous" to the intact (non-deleted) sequence and vice versa. All the sequences present in the subject were derived from a common ancestor. Employing the application's definition here, the nucleic acid lacking the 20 bp was "not naturally present in the genome" of other viruses without the deletion. The nucleic acid of the virulent form of HIV-1 in this subject "was not naturally present" in the deleted nucleic acid of the attenuated variant. Yet, it would have been considered absurd had we labeled these sequences as heterologous. Similarly, the Patent Office's calling the CB4-P and CB4-V viruses heterologous is scientifically totally untenable.

8. In contrast to the discussion in Section 7, above, examples of sequences from related viruses that are nonetheless considered heterologous to HIV-1 are sequences from the viruses HIV-2 and SIV (simian immunodeficiency virus). Both HIV-2 and SIV are considered by the virology community to be distinct viruses from HIV-1 and vary from each other by more than 35% at the nucleotide level. Different primers are needed to amplify these different genomes. The chimeric virus composed of portions of HIV-1 and SIV, known now as the SHIV (engineered by Sodroski and colleagues for use primarily in vaccine design), expresses the *env* gene of HIV-1 yet can still infect and replicate in rhesus macaques (the natural host of SIV but not of HIV-1). This recombinant, chimeric virus, ***like*** **the recombinant chimeric coxsackieviruses** of the above-identified invention, but ***unlike*** **the CB4-V and CB4-P** viruses of the Caggana reference, is definitely considered to contain heterologous sequences.

9. In summary, I reiterate that the Patent Office has adopted here a meaning of ***heterologous*** polypeptides and nucleic acid sequences that is fundamentally different from the meaning accepted by the scientific community and from that in the application. The Caggana reference describes replacement of a few, subtly varying homologous nucleotide sequences in the laboratory generation of an attenuated CB4 variant from an extremely closely related virulent CB4 variant. Based upon my knowledge of an extensive literature dealing with viral variation and my expertise in this very field, I believe that no virologist would consider the altered sequences

described in the Caggana reference to be heterologous. Considering both the present patent application and what is accepted in the field, I can see no proper basis for the Patent Office's conclusions about what this reference says. Likewise, I do not see any connection between the reference and the Applicants' claims.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

5/25/05 Barbara Weiser

Date Barbara Weiser

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5/25/05 Barbara Weiser

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# CURRICULUM VITAE
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## Education

Vassar College, A.B. in English, 1969
SUNY Stony Brook, graduate study in Cell Biology, 1970-71
University of Pittsburgh School of Medicine, M.D., 1975

## Postdoctoral Training

Intern and Resident in Internal Medicine, Bellevue Hospital-New York University Medical Center, 1975-78
Infectious Diseases Fellow, Memorial Sloan-Kettering Cancer Center, 1978-80
Postdoctoral Research Fellow, Laboratory of Dr. Harold Varmus, Department of Microbiology, University of California at San Francisco, 1980-83
Postdoctoral Research Fellow, Laboratory of Dr. William Robinson, Infectious Disease Division, Department of Medicine, Stanford University, 1984-85

## Staff Appointments

Instructor of Medicine, Cornell University Medical College, Khymer Refugee Project, Khao-I-Dang Holding Center, Thailand, June-July, 1980
Assistant Professor of Medicine, Division of Infectious Diseases, San Francisco General Hospital, University of California at San Francisco, January, 1981
Assistant Professor of Medicine and Microbiology, SUNY Stony Brook, 1985-91
Co-Director of HIV Research, Wadsworth Center, New York State Department of Health, 1991-present
Associate Professor of Medicine, Divisions of HIV Medicine and Infectious Diseases, Albany Medical College, 1991-1998
Professor of Medicine, Albany Medical College, 1998-present

## Board Certification

Diplomate, American Board of Internal Medicine, 1978

## Honors

American Cancer Society Postdoctoral Research Fellowship, 1981-1982
National Heart, Lung and Blood Institute Clinical Investigator Award, 1982-1987
Mary Pangborn Award for Research with an Impact on Clinical Medicine, New York State Department of Health, 1997

## Patent

US Patent: "Analysis of HIV-1 Co-Receptor Utilization in the Clinical Care of HIV-1-Infected Patients," patent number US 09/963,064," 2003

## Present National Committee Membership

NIH Women's Interagency HIV Study (WIHS) Laboratory Committee, 1993-present (Chair of Subcommittees on Virology, 1994-98, and HIV Infection in Drug-Using Women, 1995-98)
WIHS Scientific Advisory Board, 2002-present
Chair, WIHS Scientific Working Group on HIV Virology, 2002-present

## Present Local Committee Membership

Wadsworth Center Biosafety Committee, 1995 - present

## Past Committee Membership

National Institutes of Health Committees
AIDS Clinical Trial Group (ACTG) Basic Research Committee, 1987-90
ACTG Virology Committee, 1987-90
AIDS Related Research Ad Hoc Study Sections, 1989-91 and 95-96
NIAID AIDS Research Review Committee, Basic Science I, 1991-1994
WIHS Review Team, 1995-1996
WIHS Executive Committee, 1997-2002

Institutional Committees
SUNY Stony Brook Biomedical Research Support Committee, 1988-91
SUNY Stony Brook Biotechnology Center Committee, 1988-91
Wadsworth Center Tuberculosis Committee, 1992
Wadsworth Center Axelrod Institute Space Allocation Committee, 1993
Wadsworth Center Axelrod Symposium Committee, 1994
Wadsworth Center Peer Review Board, 1995-1998
Chairperson, Albany Medical Center Search Committee for the Chief of the Division of HIV Medicine, 1999-2000

## Membership in Professional Societies

American Society for Microbiology
American Association for the Advancement of Science
Infectious Diseases Society of America
HIV Medicine Association
International AIDS Society
New York Society of Infectious Diseases

## Conference Organizer

National Conferences on Women and HIV, Los Angeles, 1997 and 1999
Satellite Meeting on HIV Infection in Women, 4th Conference on Retroviruses and Opportunistic Infections, Washington, DC, 1997

## Ad Hoc Reviewer

Virology
AIDS
Journal of Virology
Clinical Infectious Diseases
Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology

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Philpott S, Burger H, Tsoukas C, Foley B, Anastos K, Kitchen C, Weiser B. (2005) Human immunodeficiency virus type 1 genomic RNA sequences in the female genital tract and blood: compartmentalization and intrapatient recombination. *J Virol*, 79:353-63.

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## Patent

US Patent: "Analysis of HIV-1 Co-Receptor Utilization in the Clinical Care of HIV-1-Infected Patients," patent number US 09/963,064," 2003

## Present National Committee Membership

NIH Women's Interagency HIV Study (WIHS) Laboratory Committee, 1993-present (Chair of Subcommittees on Virology, 1994-98, and HIV Infection in Drug-Using Women, 1995-98)
WIHS Scientific Advisory Board, 2002-present
Chair, WIHS Scientific Working Group on HIV Virology, 2002-present

## Present Local Committee Membership

Wadsworth Center Biosafety Committee, 1995 - present

## Past Committee Membership

National Institutes of Health Committees
AIDS Clinical Trial Group (ACTG) Basic Research Committee, 1987-90
ACTG Virology Committee, 1987-90
AIDS Related Research Ad Hoc Study Sections, 1989-91 and 95-96
NIAID AIDS Research Review Committee, Basic Science I, 1991-1994
WIHS Review Team, 1995-1996
WIHS Executive Committee, 1997-2002

Institutional Committees
SUNY Stony Brook Biomedical Research Support Committee, 1988-91
SUNY Stony Brook Biotechnology Center Committee, 1988-91
Wadsworth Center Tuberculosis Committee, 1992
Wadsworth Center Axelrod Institute Space Allocation Committee, 1993
Wadsworth Center Axelrod Symposium Committee, 1994
Wadsworth Center Peer Review Board, 1995-1998
Chairperson, Albany Medical Center Search Committee for the Chief of the Division of HIV Medicine, 1999-2000

## Membership in Professional Societies

American Society for Microbiology
American Association for the Advancement of Science
Infectious Diseases Society of America
HIV Medicine Association
International AIDS Society
New York Society of Infectious Diseases

## Conference Organizer

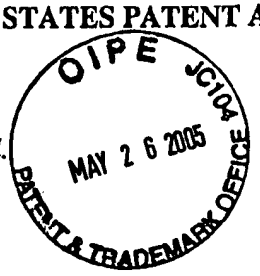
National Conferences on Women and HIV, Los Angeles, 1997 and 1999
Satellite Meeting on HIV Infection in Women, 4th Conference on Retroviruses and Opportunistic Infections, Washington, DC, 1997

## Ad Hoc Reviewer

*Virology*
*AIDS*
*Journal of Virology*
*Clinical Infectious Diseases*
*Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology*

Atty Dkt: 29025.0001

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**




In the application of:

Arlene RAMSINGH *et al.*

Serial No.: 09/879,572

Filing Date: June 12, 2001

For: COXSACKIEVIRUS B4 EXPRESSION VECTORS AND USES THEREOF

Examiner: Stacy Chen

Group Art Unit: 1648

Atty Dkt: 29025.0001

Customer No.

26694

PATENT TRADEMARK OFFICE

**DECLARATION OF GEORGE F. VANDE WOUDE, PH.D.**
**PURSUANT TO 37 C.F.R § 1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am an expert in the field of virology, molecular biology and particularly molecular oncology. My work over the past 30+ years has focused on oncogene research. I have worked with various families of viruses and began my post-graduate career studying foot-and-mouth disease virus (FMDV), a member of the picornavirus family just like coxsackieviruses. I am an author/co-author of about 300 peer-reviewed publications, review articles and book chapters in my field. I have been the Director of the Van Andel Research Institute in Grand Rapids, MI, since 1999. Before that I was the Director of the Division of Basic Sciences of the National Cancer Institute (NCI), NIH, Bethesda, MD and Head of the Molecular Oncology Section, at the NCI-Frederick Cancer Research and Development Center in Frederick, MD (the latter position dating back to 1983. I was a section head and laboratory head at the NCI in Bethesda starting in 1972. I was elected to the National Academy of Sciences in 1993 and have been the recipient of numerous awards and honors. I have been and continue to be a member of numerous scientific review committees and advisory boards of research institutions around the world. My *Curriculum Vitae* is attached. I am

an inventor/co-inventor of a seven U.S. Patents, various corresponding foreign patents and numerous pending U.S. and foreign patent applications.

2. I was asked to reviewed relevant sections of the patent application identified above, the outstanding Office Action and the rejected claims due to my expertise in molecular genetics, virology and oncology,. My comments are based on my knowledge of the genetics of RNA viruses, DNA viruses, and mammalian cells. I am familiar with coxsackieviruses, and I spent several years working on a related virus, FMDV.

3. The claims I reviewed concern recombinant attenuated coxsackievirus B4 (CB4) virions engineered to express a foreign (or heterologous) sequence. I say "foreign" because the sequence is, or can be, derived from a different source, not a coxsackievirus of the same serotype or strain, but, for example, a truly **different** virus (such as HIV). The application also describes the use of nucleotide sequences from a variety of non-viral pathogens that encode various bacterial, parasitic and cellular, including mammalian, proteins. The CB4 virions described in the claims are genetically modified with an inserted foreign (heterologous) nucleic acid sequence that encodes a foreign (heterologous) polypeptide or peptide that can be expressed as a viral capsid fusion of the CB4 protein VP1 or as an upstream amino-terminal fusion of the CB4 protein VP4 . A major emphasis of the invention is the use of these recombinant attenuated CB4 virions as "vaccine vectors" to induce an immune response to the foreign peptide antigen engineered into CB4 virions.

4. The Patent Office's analysis of the paper by Caggana *et al. J Virol. 67*:4797-803 (1993), which was used to reject the claims on the grounds that the claims were anticipated by this paper, is incorrect as I interpret their argument. The rejection asserts that the two viral variants discussed in the paper, CB4-V and CB4-P, are "heterologous." This interpretation is improper because viral variants such as the above two are not foreign or heterologous. Genetically they are two alternative sequences of the same gene -- "alleles" or "allelic variants." I know of no definition of "foreign" or "heterologous," including the definition appearing in the application, that would result in the CB4-P and CB4-V nucleic acid sequences (or their protein products or the viruses which carry them) being called foreign or heterologous to any CB4 virus.

5. As I understand it, the Patent Office has said that it considers the sequences in Caggana *et al.* to be heterologous because "the P1 region of CB4-V was not naturally present in the genome of the CB4-P virus." Whatever this statement means, these two variants cannot be viewed as different viruses. The Patent Offices states that the Caggana reference describes a "heterologous nucleic acid **inserted** into an open reading frame that results in fusion to the capsid protein of the virion." This is backwards: the CB4-P and V sequences are "**homologous,**" the converse of "**heterologous**". Stated correctly, a portion of the nucleic acid of a CB4 virus "replaces" a *homologous* portion of a nucleic acid of the same length to yield the other variant. "Insertion" of a sequence requires the addition of coding capacity - not present in Caggana, but characteristic of the present invention where foreign sequences are inserted so that a CB4 virion expresses *de novo* a foreign peptide such as an ovalbumin peptide or various HIV peptides.

6. For the reasons cited above, I respectfully disagree with the Patent Office's conclusion that Caggana *et al.* describes "a CB4 virion with *heterologous* nucleic acid *inserted* into an open reading frame that results in fusion to the capsid protein of the virion". That is not an accurate characterization of what is described by this reference.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

May 26, 2005 George F. Vande Woude

Date

**GEORGE F. VANDE WOUDE, Ph.D.**
Director, Van Andel Research Institute

## EDUCATION

Ph.D., Rutgers University, 1964
M.S., Rutgers University, 1962
B.A., Hofstra University, 1959

## PROFESSIONAL BACKGROUND

| | |
|---|---|
| 1999-present | Director, Van Andel Research Institute, Grand Rapids, MI |
| 1998 - 1999 | Director, Division of Basic Sciences, National Cancer Institute, NIH, Bethesda, MD |
| 1995 - 1998 | Scientific Advisor to the Director for Basic Sciences, National Cancer Institute, NIH, Bethesda, MD |
| 1983 - 1998 | Director, ABL-Basic Research Program; Head, Molecular Oncology Section, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD |
| 1981 - 1983 | Chief, Laboratory of Molecular Oncology, National Cancer Institute, NIH, Bethesda, MD |
| 1972 - 1981 | Head, Human Tumor Studies Section, Viral Biology Branch, National Cancer Institute, NIH, Bethesda, MD |
| 1965 - 1972 | Research Chemist, U.S. Department of Agriculture, Plum Island Animal Disease Laboratory, Greenport, NY |
| 1964 - 1965 | Postdoctoral Research Associate, U.S. Department of Agriculture, Plum Island Animal Disease Laboratory, Greenport, NY |
| 1960 - 1964 | Research Assistant, Rutgers University, New Brunswick, NJ |

## HONORS, AWARDS, PROFESSIONAL RECOGNITION

U.S. Public Health Service Predoctoral Trainee, Rutgers University, 1960-1964
U.S. Department of Agriculture Postdoctoral Research Associateship Plum Island Animal Disease Laboratory, 1964-1965
Recipient, NIH Merit Award, 1982
Recipient, 1989 Pasarow Foundation Award for Cancer Research
Hassel Foundation 1990 George Khoury Memorial Lecture, Philadelphia, PA
Recipient, 1992 Lifetime Achievement Award in Technology Transfer, NASA
Honorary Doctorate of Science, Michigan State University, 1999
Grand Rapids Magazine Medical Hall of Fame Inductee, 2001
Member, National Academy of Sciences, 1993-present
Section Chair, Medical Genetics, Hematology & Oncology, National Academy of Sciences, 2004-present
Fellow, American Academy of Microbiology, 1997-present
Member, American Association for Cancer Research, 1984-present
Member, Board of Directors, American Association of Cancer Institutes, 2003-present
Member, Advisory Council, General Motors Cancer Research Foundation, 1994-present
President and CEO, Core Technology Alliance Corporation, 2004-Present

Collaborating Partner, National Dialogue on Cancer, 2000-present

Executive Board Member, Grand Rapids Clinical Oncology Program, Grand Rapids, MI, 1999-present

Alice Hogge and Arthur A. Baer Professorship and Visiting Professor, Department of Radiation and Cellular Oncology, University of Chicago, 2002-2004

Adjunct Professor, Johns Hopkins School of Medicine, Baltimore, 1985-1997

Member, Board of Directors, American Assoc for Cancer Research, 2001-2004

Member, American Association for Cancer Research's Science Policy and Legislative Affairs Committee, 2003

Kovalenko Medal Award Committee, American Association for Cancer Research, 2003

Co-Chair, Laboratory Research Awards Committee, American Association for Cancer Research, 2003

Rhoads Memorial Award Committee, American Association for Cancer Research, 2000-2001

Visiting Scientific Advisory Committee, Columbia University Comprehensive Cancer Center, New York, NY, 1986-2004

Board of Scientific Advisors, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA, 1995-present

External Advisory Committee, Karmanos Cancer Institute & Prentis Comprehensive Cancer Center at Wayne State University, Detroit, MI, 2001-present

Scientific Advisory Panel, North Shore-Long Island Jewish Research Institute, Manhasset, NY, 2002-present

Committee of Scientific Advisors, U.S. Military Cancer Institute, Washington, DC, 2001- present

Chair, External Advisory Board, Children's Cancer Research Institute, University of Texas Health Science Center, San Antonio, TX, 2003-present

Member, Governing Board of Directors, Biosciences Research & Commercialization Center, Western Michigan University, Kalamazoo, 2004-present

Advisory Board, Natl Cancer Legislation Advisory Committee/C-Change, 1999-2001 Advisory Board, Innovation Center, Michigan Life Sciences Corridor, 2000-2001

Awards Assembly, General Motors Cancer Research Foundation, 1990 -1994

National Academy of Sciences Research Briefing Panel on Oncogenes, 1984

Scientific Advisory Board of the Leonard and Madlyn Abramson Family Cancer Research Institute, University of Pennsylvania Cancer Center, 1999-2004

Board of Scientific Consultants, Memorial Sloan-Kettering Cancer Center, 1990-2002

External Advisory Committee, University of Wisconsin Comprehensive Cancer Center, Madison, WI, 1999-2002

Research Advisory Board of the Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, 1991-2000

Board of Scientific Advisors, Univ of Pennsylvania Cancer Center, 1984-1999

Scientific Advisory Committee, Vincent T. Lombardi Cancer Research Center, Georgetown University, Washington, DC, 1988-1999

Board of Scientific Counselors, Division of Cancer Etiology, National Cancer Institute, NIH, Bethesda, MD, 1985-1989

Int'l Advisory Committee, Maimonides Conferences on Cancer Research,1985-1988

Scientific Advisory Committee and Board of Managers of the Wistar Institute, Philadelphia, PA, 1985-1991

Member, Scientific Advisory Board, St. Jude Children's Hospital, 1986-1995

Scientific Advisory Board, Israel Cancer Research Fund, 1986-1990

Chairman, Board of Scientific Advisors, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, 1987-1990

Leukemia Society of America Grant Review Subcommittee, 1987-1991

External Advisory Committee, Utah Reg Cancer Center, Salt Lake City, 1988-1994

International Review Panel of the Medical Research Council, Ottawa, Canada, 1996

## EDITORIAL BOARDS / CONFERENCE ORGANIZATION

Editorial Board, *DNA and Cell Biology*, 1984-present
Co-editor (with George Klein), *Advances in Cancer Research*, 1987-present
Editorial Board, *Oncogene*, 1987-present
Member, Editorial Board, *Molecular Imaging*, 2001-present
Member, Editorial Board, *Cell Cycle*, 2001-present
Member, Editorial Board, *Molecular Cancer Research*, 2003-present
Member, Editorial Board, *Cancer Genomics and Proteomics*, 2003-present
Guest Editorial Member, *Japanese Journal of Cancer Research*, 1987-2004
Member, Editorial Board, *Cancer Letters*, 2000-2004
Research Advisory Panel, *Advances in Oncology*, 1994-2001
Associate Editor, *Cancer Research*, 1997-2000
Editor, *Journal of Virology*, 1980-1990
Associate Editor, *Cell*, 1984-1990
Founding Editor and Editor-in-Chief, *Cell Growth and Differentiation*, 1989-1996
Editorial Board, *Leukemia*, 1993-1996
Founder and President, Foundation for Advanced Cancer Studies, 1984-present
Organizer and Sponsor, Annual Oncogene Meetings, Frederick, MD, 1985-2004

## PROFESSIONAL SOCIETY MEMBERSHIPS

Sigma Xi
American Chemical Society
American Society for Microbiology
American Association for Advancement of Science
American Association for Cancer Research
American Society for Cell Biology
American Academy of Microbiology

## LIST OF PUBLICATIONS


1. Vande Woude GF: A new twist to DNA. Chem and Eng News, July 1961 (Letter to the Editor).
2. Vande Woude GF and Davis FF: Barrier electrophoresis: A new electrophoretic technique. Anal Biochem 6:240-250, 1963.
3. Vande Woude GF and Davis FF: Fractionation of histones in polyacrylamide gels. Anal Biochem 12:444-451, 1965.
4. Arlinghaus R, Polatnick J, and Vande Woude GF: Studies on foot-and-mouth disease virus ribonucleic acid synthesis. Virology 30:541-550, 1966.
5. Vande Woude GF, Trautman R, and Bachrach HL: The influence of the host cell, ionic strength and chymotrypsin of foot-and-mouth disease virus electrophoretic mobility. Arch Ges Virusforsch 20:71-80, 1967.
6. Vande Woude GF: Inactivity of foot-and-mouth disease virus at ionic strength dependent isoelectric points. Virology 31:436-441, 1967.
7. Vande Woude GF, Arlinghaus RB, and Polatnick J: Inhibition of ribonucleic acid methylation in the foot-and-mouth disease virus host cell. Biochem Biophys Res Commun 29:483-489, 1967.
8. Vande Woude, GF and Bachrach HL: Evidence for a single structure polypeptide in foot-and-mouth disease. Arch ges Virusforsch 23:353-356, 1968.
9. Polatnick J, Vande Woude GF, and Arlinghaus RB: Changes in protein and nucleic acid metabolism in baby hamster kidney cells infected with foot-and-mouth disease virus. Arch ges Virusforsch 23:218-226, 1968.
10. Bachrach HL and Vande Woude GF: Amino acid composition and C-terminal sequence of foot-and-mouth disease virus protein. Virology 34:282-289, 1968.
11. Ascione R and Vande Woude GF: Inhibition of host cell ribosomal ribonucleic acid methylation by foot-and-mouth disease virus. J Virol 4:727-737, 1969.
12. Vande Woude GF: Biochemical investigation of foot-and-mouth disease virus. International Pilot Conference of Foot-and-Mouth Disease Virus, New York, NY, 1969.
13. Vande Woude GF, Polatnick J, and Ascione R: Foot-and-mouth disease virus- induced alterations of baby hamster kidney cell macromolecular biosynthesis: Inhibition of ribonucleic acid synthesis. J Virol 5:458-463, 1970.
14. Vande Woude GF and Bachrach HL: The number and molecular weight of foot- and-mouth disease virus capsid proteins and the effects of maleylation. J Virol 7:250-259, 1971.
15. Ascione R and Vande Woude GF: Ribosomal factors effecting the stimulation of cell-free protein synthesis in the presence of foot-and-mouth disease virus ribonucleic acid. Biochem Biophys Res Commun 45:14, 1971.
16. Bachrach HL, Vande Woude GF, and Swaney JB: Structure and properties of FMDV and of virus-specific components. Proceedings of the Second International Congress of Virology, Budapest, 1971.
17. Ascione R, Vande Woude GF, Bachrach HL, and Ehrenfeld, E: Protein biosynthesis in cellular and cell-free systems directed by the ribonucleic acid from foot-and-mouth disease virus. Proceedings of the Second International Congress for Virology, Budapest, 1971.
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21. Swaney JB, Vande Woude GF, and Bachrach HL: Sodium dodecyl-sulfate- dependent anomalies in gel electrophoresis: Alterations in the banding patterns of foot-and-mouth disease virus polypeptides. Anal Biochem 58:337-346, 1974.
22. Vande Woude GF and Ascione R: Translation products of foot-and-mouth disease virus-infected baby hamster kidney cells. Arch ges Virusforsch 45:259-271, 1974.
23. Andrese AP, Vande Woude GF, and Walling MJ: Ultra-structural study of herpes simplex virus infection of mouse cells chronically producing Rauscher leukemia virus. J Natl Cancer Inst 53:1169-1173, 1974.
24. Holder WG, Robery WG, and Vande Woude GF: Activation of a C-type virus from the human carcinoma cell line HBT-3 by iododeoxyuridine and testosterone. Nature 249:759-762, 1974.

25. Smida J, Smidova V, Andrese A, and Vande Woude GF: Search for oncogenicity of type C particles released from mammalian cell lines transformed by avian oncornaviruses. Neoplasma 21:609-618, 1974.
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30. Vande Woude GF, Robey WG, Oskarsson MK, Haapala DK, Fischinger PJ, Naso RB, and Arlinghaus RB: Properties of Moloney sarcoma virus-specific p60 and its detection in transformed cells. Bibl Haemat (Basel) 43:125-127, 1976.
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simplex virus type 1 cloned DNA fragments. Virology 103:228-231, 1980.

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53. Blair DG, Oskarsson M, McClements L, and Vande Woude GF: The long terminal repeat of Moloney sarcoma provirus enhances transformation. In Haematology and Blood Transfusion, Modern Trends in Human Leukemia IV, Vol. 26. R Neth, RC Gallo, T Graaf, K Mannweiler and K Winkler (eds). Springer-Verlag, Berlin, pp. 400-466, 1981.
54. McClements WL and Vande Woude GF: Cloning retroviruses: Retrovirus cloning? Genetic Engineering 3:89-107, 1981.
55. Blair DG, Oskarsson MK, Wood TG, McClements WL, Fischinger PJ, and Vande Woude GF: Activation of the transforming potential of a normal cell sequence: A molecular model for oncogenesis. Science 212:941-943, 1981.
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57. Straus, SE, Aulakh HS, Ruyechan WT, Hay J, Casey TA, Vande Woude GF, Owens J, and Smith HA: Structure of Varicella-Zoster virus DNA. J Virol 40:516-525, 1981.
58. Levinson, B, Khoury G, Vande Woude GF, and Gruss P: Activation of the SV40 genome by the 72 base-pair tandem repeats of Moloney sarcoma virus. Nature 295:568-572, 1982.
59. Watson R, Oskarsson M, and Vande Woude GF: Human DNA sequence homologous to the transforming gene (mos) of Moloney murine sarcoma virus. Proc Natl Acad Sci USA 79:4078-4082, 1982.
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Arlene RAMSINGH *et al.*

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Examiner: Stacy Chen

Group Art Unit: 1648

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Customer No. 26694

PATENT TRADEMARK OFFICE

## 3RD DECLARATION OF ARLENE I. RAMSINGH PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, the undersigned, declare as follows:

1. I am a co-inventor of the present application. My *Curriculum Vitae* and background have already been submitted as part of an earlier Declaration. It is my intention here to help clarify the record in this case by recounting some relevant history of Coxsackievirus B4 (CB4) viruses and the variants that are described in this application, well-known in the art, and that are intended to be used in the claimed invention.

2. The prototypic strain of CB4, JVB, was isolated in the 1950's and deposited in the ATCC by Sickles and Daldorf from the institute which was the forerunner of my place of employment, the Wadsworth Center of the New York State Dept. of Public Health. This deposited virus was given ATCC # VR-184 and remains publicly available. It can be found by entering this number on the ATCC virus search page

http://www.atcc.org/common/catalog/animalVirology/animalVirologyIndex.cfm .

3. Virus derived from the deposited JVB strain has been sequenced at least twice, first by Jenkins and colleagues (Jenkins *et al.* 1987[1]) and then by my lab - half the genome - from the 5' end (Ramsingh *et al.* 1992[2]). The latter sequence is also in GenBank, Accession #S39291 (see: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=250908). The sequence differences between CB4 viruses are discussed below.

4. Origin of the CB4-P designation: I was given the JVB strain of CB4 by a colleague, R. Diebel, at the Wadsworth Center in 1989 and "renamed it CB4-P" to stand for the prototype CB4 virus which was intended to distinguish it from its virulent variant, CB4-V. Thus CB4-P is JVB, despite the fact that the sequences are not identical, as explained below. A paper by my group published in 1989[3] was the first to describe what we now call "CB4-P." This paper states at p. 349 that Coxsackie B4 (JVB) was kindly provided by R. Diebel (Wadsworth Center) On p. 350, it states that the prototypical CB4 (JVB) is designated CB4-P. The (-P) designation distinguishes this virus from a virulent one designated CB4-V. CB4-P was not to be distinguished from JVB - rather I considered it the same and merely renamed it.

5. Random Variation/Spontaneous Mutation in Viruses: As virus populations are propagated, they inexorably accumulate random mutations and begin to diverge somewhat from the "parental" sequence. RNA virus populations are constantly changing due to the lack of editing function in the enzyme that replicates their genes, RNA-dependent RNA polymerase. Thus, RNA virus populations are "plastic" and this plasticity leads to the ongoing generation of genetic variants within a strain. See Declaration of Steven Tracy (submitted concurrently). Many of these mutations are ***neutral*** either because they do not result in amino acid changes, or, if they do, the change does not affect function so there is no selection for or against them. The net result is that if the original deposited JVB virus from the 1950's had been sequenced and compared to the JVB virus that the ATCC would have sent out in 1987 or 1992 or 2004 under the same ATCC #, some changes in nucleotide, as well as in amino acid, sequence would have been inevitable as a result of

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[1] Jenkins *et al.* 1987. "The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the picornaviridae." *J. Gen. Virol. 68*:1835-1848)

[2] Ramsingh *et al.* 1992. "Identification of candidate sequences that determine virulence in Coxsackievirus B4." *Virus Res 23*:281-292

[3] Ramsingh *et al.* 1989. "Severity of disease induced by a pancreatropic Coxsackie B4 virus correlates with the H-2Kq locus of the major histocompatibility complex. *Virus Res 14*:347-358

growing the virus (in the ATCC lab or the recipient's lab). Such virus, if stored and then retested (sequenced) years later (even under the same strain designation) would have those few mutations generated during the previous rounds of cultivation. This fully explains the nucleotide sequence differences between JVB in the virus population of Jenkins et al. (*supra)* and CB4-P (Ramsingh et al., 1992, *supra*). Unless the environment in which the virus was passaged had been radically changed (different cell types, temperatures, multiplicities-of-infection, etc.), one would expect to find a virus (in 2004, for example, but derived from the much earlier ATCC deposit) to be very closely related, with only a small number of changes in the sequence. The virus population at these two points in time would be considered the same virus (=the same viral strain).

6. Sequence Differences: The two sequences for the JVB/CB4-P virus were found to be ever so slightly different. Again, this variability arose from random mutation during passage.

(A) A comparison of the amino acid sequences of several picornaviruses (the class to which coxsackieviruses belong) performed by Jenkins *et al. (supra)* showed that the P1 region encoding the viral polyprotein (and, through it, the four viral capsid proteins) was the most divergent. Comparison of the P1 regions of two distinct **serotypes** of the Coxsackie group B viruses, CB3 and CB4, showed 77.9% nucleotide sequence identity. CB4 and CB3 are *different* virus populations (despite the high degree of sequence identity).

(B) A comparison of the sequences of the P1 regions of the JVB virus reported by Jenkins *et al. (supra)* and the CB4-P virus (also JVB), reported by Ramsingh et al., 1992, *supra*, reveals 99.8% at the nucleotide level and 99.6% at the amino acid level. Therefore, CB4-P and the JVB are essentially the same virus population. In a stretch of almost 3300 nt's, that includes the P1 region - the most divergent/variable part of the viral genome - there were only 9 total nucleotide substitutions (4 non-coding and 5 coding regions). Two of the coding region mutations were silent whereas the other 3 resulted in amino acid substitutions which did not affect viral function (and bear **no relationship** to the site of, or nature of, the genetic manipulations of the present invention). This variation is exceedingly small so that two such sequences would never be considered "heterologous".

If I were to fully sequence nucleic acid of the CB4-P virus stock I have stored in my lab now (with or without further propagation), there would likely be some random variation in sequence from the same virus that we sequenced in 1992. It would still be the same virus (or a genetic equivalent). The reasons for this were explained above. It is my practice to always verify the sequence of the relevant region of the virus (where heterologous DNA is to be inserted) when preparing a new virus batch. Those skilled in the art would do the same, thereby confirming that they have the same virus.

7. The current Office Action states that CB4-P is "required to practice the claimed invention" which I understand to mean that the invention cannot be practiced by another person unless that person is handed the CB4-P virus that I used. This leads the Patent Office to conclude that the application does not adequately support such use without a Patent Deposit of this virus. This statement and conclusion is not accurate for several reasons.

(A) Between the application and the publicly available sequence information, including that in Jenkins *et al., supra*; Ramsingh et al., 1992, *supra* and Caggana *et al.*, 1993, (which is part of record of this case and cited in the present Office Action), a person of ordinary skill in the field can routinely make, test (verify) and use the genetically engineered virions (or nucleic acid molecules) of the claims - that comprise an exogenous, foreign, heterologous nucleotide sequence inserted in the indicated sites of the P1 region of the viral genome. The person can start with any CB4 virus that is described in the application or known in the field, and *if desired*, can modify the viral nucleic acid sequence to make it match up perfectly with the CB4-P virus that I described in the application and whose sequence I reported years earlier. However, importantly, as would be appreciated by a person of skill in this field, there is no reason the starting virus would have to match up perfectly over its entire genome. It just needs to have the correct sequences at the sites used to introduce the heterologous nucleic acid, and that is described in detail in the specification.

(B) Technologically speaking, any virus that is a genetic equivalent of CB4-P could readily be used to practice the claimed invention. The point of genetic equivalence is important because we are dealing here with RNA viruses. As noted in Section 5, above, and in the accompanying Tracy Declaration, RNA virus populations are constantly changing. CB4-P is one genetic variant of the JVB strain of CB4. Genetic variants of a strain are genetically

equivalent viruses because they share a high degree of sequence identity. As an example, the nucleotide sequence of the most divergent region of the viral genome, P1, of CB4-P (as reported in Ramsingh *et al.* 1992, *supra* ) and that of JVB (as reported in Jenkins *et al.* *supra*) shows 99.8% sequence identity while the amino acid sequences are 99.6% identical.

8. The specification teaches insertion of heterologous nucleic acids into the genome of CB4 using CB4-P as but a convenient example. The salient point for appreciating the scope of the invention is the genetic equivalence and not the phenotypic equivalence (*i.e.*, virulent vs. less virulent or avirulent) because genetically equivalent viruses can be easily manipulated by those skilled in this field, as described in the specification, to allow insertions of heterologous nucleic acids. The deposited JVB strain of CB4 (ATCC # VR-184) is genetically equivalent to CB4-P. Any CB4 virus, including the deposited JVB strain, can be used to practice the claimed invention. The specification goes into the functional similarity between CB4-P and JVB on page 6, lines 13-15; and the equivalency of CB4-P and JVB on page 14, lines 8-15

> One example of such an attenuated B4 coxsackievirus is J.V.B. (Benschoten), ATCC reference number 184 (referred to herein as JVB). The prototype virus CB4-P is originally derived from JVB and is highly similar to JVB in nucleotide and amino acid sequence. Because of this strong conservation, the JVB virus is expected to perform as an equivalent to CB4-P in the generation and use of the viral vector described herein.

9. To conclude, the CB4 virus - - the starting material for the claimed recombinant coxsackievirus B4 virions[4] which are engineered to contain an inserted heterologous nucleic acid that encodes a heterologous polypeptide fused to a capsid protein of the virion - - is publicly available. Also available, in the form of published papers, GenBank sequences and the present application, is all the information needed to make and use the claimed virions and nucleic acid composition. On that basis, the Patent Office's position that people in the field would not be enabled to practice the invention fully is in error and the requirement that a patent deposit of "CB4-P" virus be made is unnecessary.

[4] and for the claimed nucleic acid molecules

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

May 26, 2005 Arlene I. Ramsingh

Date Arlene I. Ramsingh

*J. gen. Virol.* (1987), **68**, 1835–1848. *Printed in Great Britain*



Key words: *coxsackievirus B4/nucleotide sequence/picornavirus*

# The Complete Nucleotide Sequence of Coxsackievirus B4 and Its Comparison to Other Members of the Picornaviridae

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SUMMARY

The genome of the prototype strain of coxsackievirus B4 (J.V.B. Benschoten) has been cloned in *Escherichia coli* and its complete nucleotide sequence determined. Excluding the poly(A) tract, the RNA genome is 7395 nucleotides in length and appears to encode a single polyprotein of 2183 amino acids. The predicted amino acid sequence of the polyprotein shows close homology (88%) to that of the previously sequenced coxsackievirus B3 and to certain regions of the polyproteins of the polioviruses and human rhinovirus 14. This allows identification of putative polyprotein cleavage signals, antigenic domains and other structural features likely to be important to the biological integrity of the virus.

## INTRODUCTION

Coxsackieviruses are members of the enterovirus genus of the family Picornaviridae. They are divided into 23 group A serotypes (CA1 to CA22, CA24) and six group B serotypes (CB1 to CB6). This antigenically diverse group of viruses has a limited host range, but exhibits a broad spectrum of tissue tropism within the natural host and is associated with a correspondingly large variety of clinical illnesses, ranging from mild respiratory infections to severe myocarditis and neurological disorders (Grist *et al.*, 1978; Melnick, 1985). The group B coxsackieviruses and especially CB4 are of particular interest in that they have been associated with type 1 juvenile onset or insulin-dependent diabetes mellitus (Yoon *et al.*, 1979; King *et al.*, 1983; Barret-Connor, 1985; Frisk *et al.*, 1985). The molecular basis of the tissue tropism of these viruses is not well understood, although there is some evidence to suggest that for the picornaviruses generally, a major determinant is the affinity of the viruses for a specific cellular receptor on the surface of the target cell (see Crowell *et al.*, 1981).

Knowledge of the primary structure and genetic organization of picornaviruses has increased dramatically in recent years, and this has provided insights into the mechanisms of replication (Kitamura *et al.*, 1981), genetic relationships (Stanway *et al.*, 1984*a*), evolution (Toyoda *et al.*, 1984), antigenicity (Minor *et al.*, 1983, 1986) and pathogenicity (Stanway *et al.*, 1984*b*; Cann *et al.*, 1984; La Monica *et al.*, 1986; Evans *et al.*, 1985). All picornaviruses share common structural features, namely an approximately 30 nm capsid of icosahedral symmetry, made up of 60 copies each of four virus-coded proteins (VP1 to VP4) enclosing a single-stranded, positive-sense RNA genome of approximately 7500 nucleotides. The RNA is polyadenylated at its 3′ terminus and has a small protein, VPg, covalently attached to the 5′ terminus. The primary translation product of picornavirus RNA is a single large polyprotein which is processed by virus-encoded proteases to yield the mature viral proteins (for a review, see Rueckert, 1985).

Recently the complete nucleotide sequence of CB3 (strain Nancy) has been determined (Lindberg *et al.*, 1987), providing detailed information on coxsackievirus genome organization and allowing a comparison at the nucleotide level with other members of the Picornaviridae.

As part of a study into the molecular basis of serotype diversity, pathogenicity and tissue tropisms of these viruses, we have determined the complete nucleotide sequence of the genome

of the prototype strain of CB4 (J.V.B. Benschoten). We provide a detailed comparison of this sequence with that of the closely related CB3 and with those of other members of the Picornaviridae. The high degree of similarity to poliovirus (PV) type 1 and rhinovirus 14 (HRV14) also allows interpretation of the predicted amino acid sequence data in relation to the three-dimensional structures recently determined for these two viruses (Hogle *et al.*, 1985; Rossmann *et al.*, 1985).

## METHODS

*Virus and cells.* Coxsackievirus B4 (strain J.V.B. Benschoten) (Dalldorf, 1950) was obtained from the American Type Culture Collection. The virus was propagated in HEp-2c cells and purified on sucrose gradients as previously described (Minor, 1980).

*Molecular cloning and nucleotide sequencing.* Purified viral RNA (approximately 2 μg) was reverse-transcribed and cloned into *Escherichia coli* JA221 by the cDNA:RNA hybrid method (Cann *et al.*, 1983; Stanway *et al.*, 1984*c*) using vector pBR322. Of the recombinants obtained, approximately 1000 were screened by hybridization using 3′-enriched and randomly primed CB4 cDNA probes (Cann *et al.*, 1983). Plasmid DNA isolated from strongly hybridizing colonies was further characterized by restriction enzyme mapping and by cross-hybridization. A set of five overlapping clones were selected which together spanned the genome (data not shown). The sequences of cDNA inserts were determined by the dideoxynucleotide method after generation of random fragments and cloning into M13mp8 as previously described (Stanway *et al.*, 1984*b*). This method was used to obtain the nucleotide sequence of the majority of the genome. The sequence of the remainder was obtained after cloning specific restriction fragments into M13mp18 or mp19. The whole of the sequence was determined at least twice and approximately 75% of it was obtained in both orientations. Throughout its assembly the sequence was compared to those of other enteroviruses and this provided a useful check on possible frameshift sequencing errors. Where significant differences were observed these were checked in the opposite orientation. The sequence data were assembled and analysed using published computer programs (Staden, 1980).

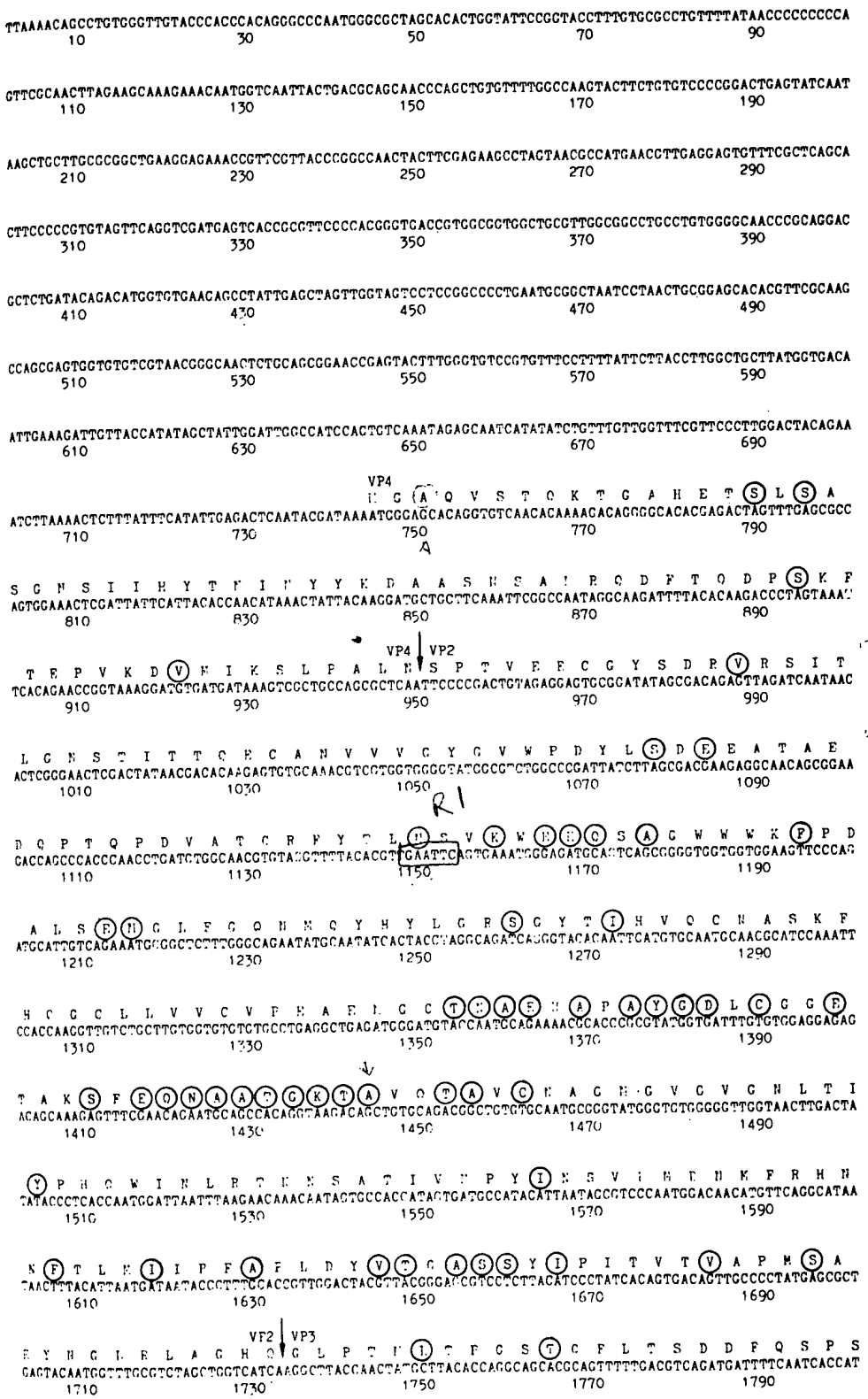
## RESULTS AND DISCUSSION

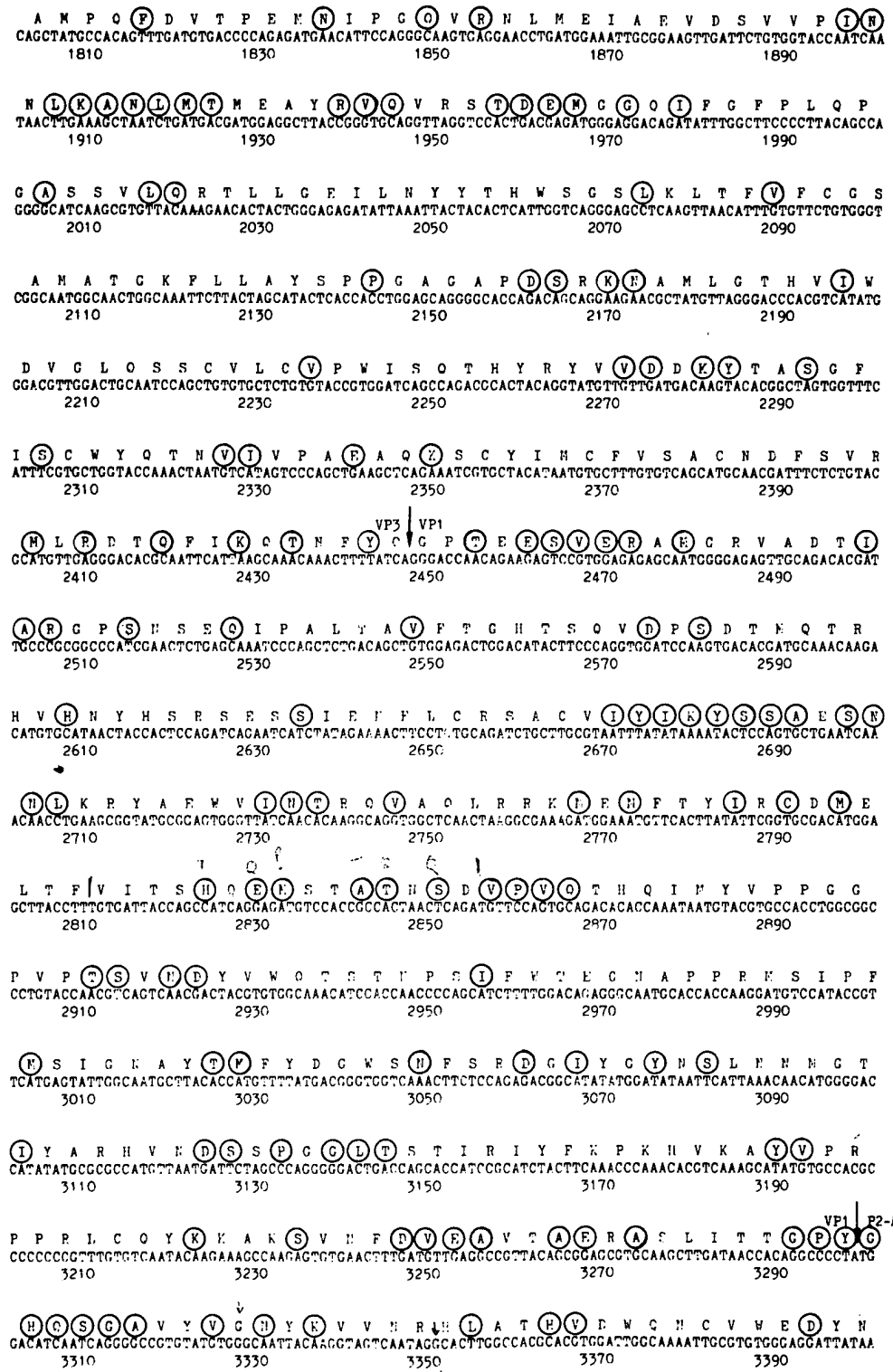
The complete nucleotide sequence and predicted amino acid sequence of CB4 (strain J.V.B. Benschoten) are shown in Fig. 1. The genome is highly homologous to those of other enteroviruses, suggesting that it has a similar genetic organization. Thus, the genome comprises a 5′ non-coding region of 743 nucleotides, a single open reading frame of 6552 nucleotides (2184 codons) and a 3′ non-coding region of 100 nucleotides prior to a poly(A) tract. Hence the total size of the CB4 genome excluding the poly(A) tract is 7395 nucleotides (compared with CB3, 7396; PV1, 7433; PV3, 7432; HRV14, 7208 nucleotides) and it has the % base composition A, 28·30; G, 24·84; C, 22·91; T, 23·95.

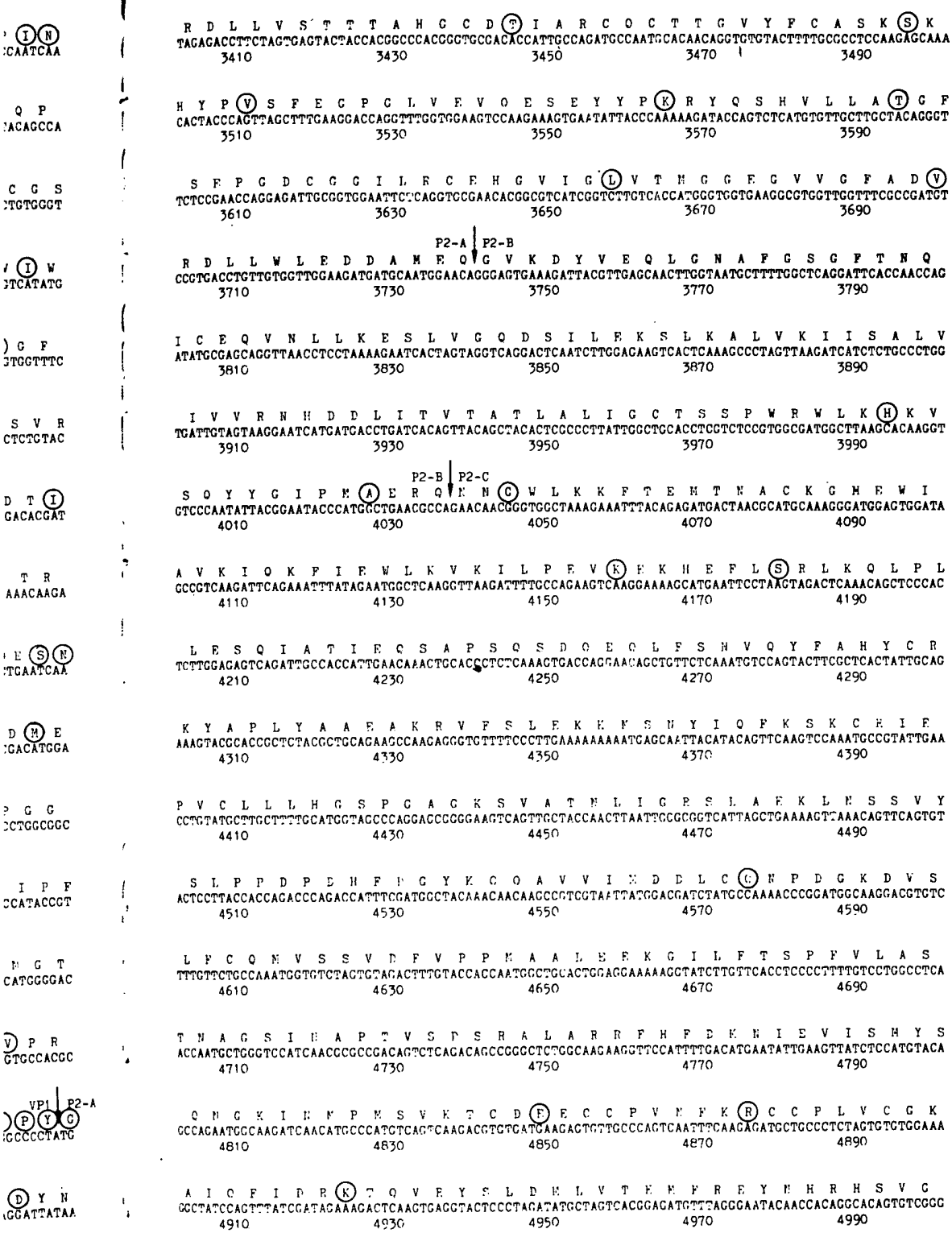
### *5′ non-coding region*

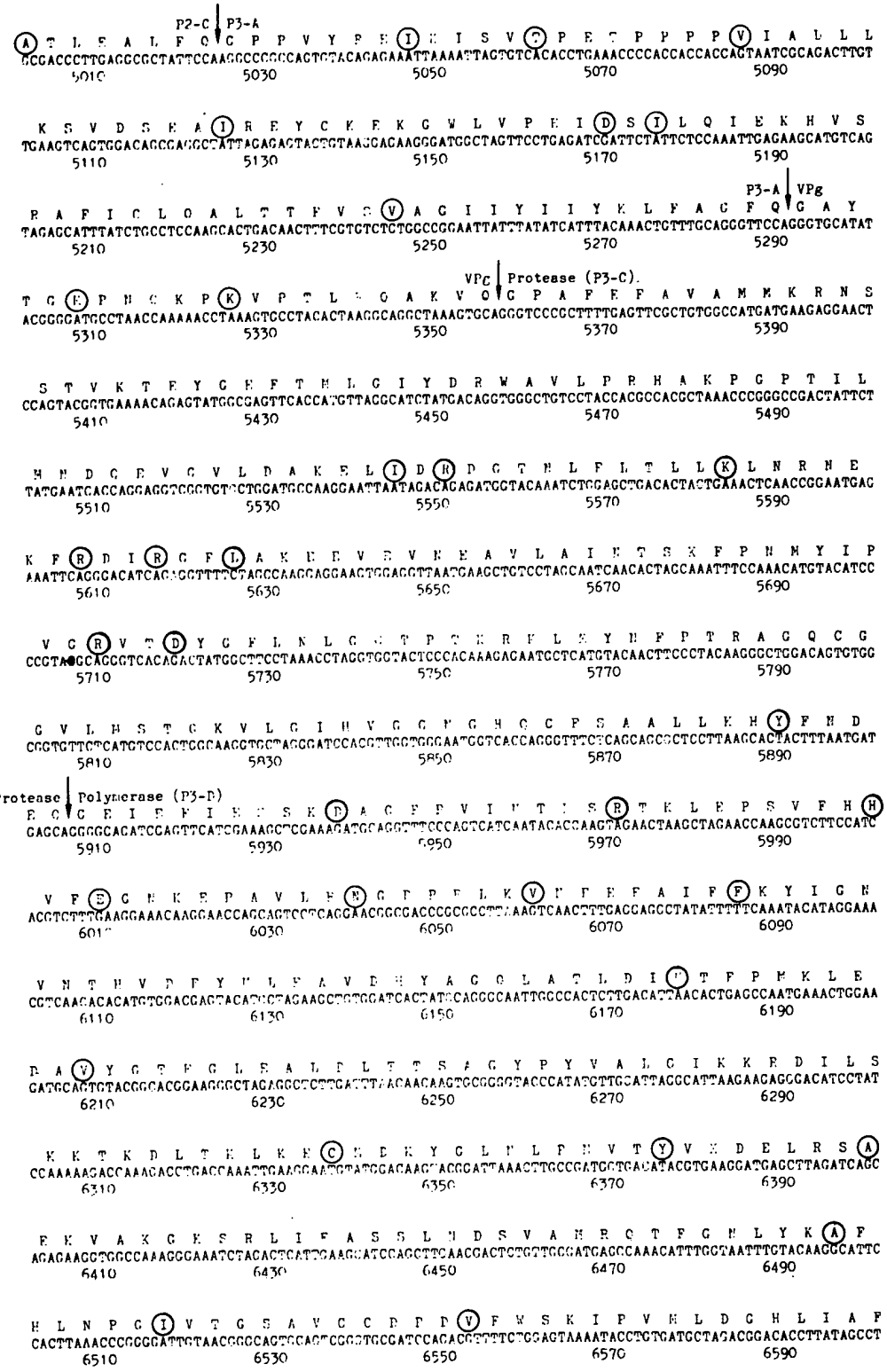
By analogy with the polioviruses, the 5′ terminal 743 nucleotides of the CB4 genome are assumed to be non-coding (Kitamura *et al.*, 1981). There are, however, seven potential translation start codons prior to that which initiates the large open reading frame at position 744. Four of these are quickly followed by stop codons. Those at positions 272 and 463 are followed by open reading frames of 66 codons and 110 codons respectively. The latter AUG is also present in CB3 and PV3 but the size of the reading frame is not conserved, being terminated after 76 amino acids at position 686 in CB3 and after 44 amino acids at position 593 in PV3. These similarities may be fortuitous though it is likely that they relate to sequence-dependent functions other than translation. It is considered unlikely that any of these short open reading frames are translated since the corresponding peptides have not been found in PV-infected cells. Furthermore, none of the AUGs have flanking nucleotide sequences favoured by eukaryotic ribosomes for the initiation of protein synthesis (Kozak, 1986).

The 5′ non-coding regions of CB4, CB3, PV1, PV3 and HRV14 can be aligned by taking into account several small deletions or insertions to give remarkable sequence homology (Table 1, Fig. 2). The first 10 nucleotides are identical in all these viruses. This conservation of sequence may be important for interactions with proteins involved in the replication of the viral RNA (Lindberg *et al.*, 1987; Hewlett & Florkiewicz, 1980; Toyoda *et al.*, 1984). Other regions of pronounced conservation include nucleotide positions 66 to 85, 446 to 472 and 547 to 567 and


VP4
VP4 VP2
R1
VP2 VP3


VP3
VP1
VP1
P2-A


R D L L V S T T T A H G C D T I A R C Q C T T G V Y F C A S K S K
TAGAGACCTTCTAGTGAGTACTACCACGGCCCACGGGTGCCACACCATTGCCAGATGCCAATGCACAACAGGTGTGTACTTTTGCGCCTCCAAGAGCAAA
3410 3430 3450 3470 3490
H Y P V S F E C P G L V E V Q E S E Y Y P K R Y Q S H V L L A T G F
CACTACCCAGTTAGCTTTGAAGGACCAGGTTTGGTGGAAGTCCAAGAAAGTGAATATTACCCAAAAAGATACCAGTCTCATGTGTTGCTTGCTACAGGGT
3510 3530 3550 3570 3590
S F P G D C G G I L R C E H G V I G L V T M G G E G V V G F A D V
TCTCCGAACCAGGAGATTGCGGTGGAATTCTCAGGTGCGAACACGGCGTCATCGGTCTTGTCACCATGGGTGGTGAAGGCGTGGTTGGTTTCGCCGATGT
3610 3630 3650 3670 3690
P2-A | P2-B
R D L L W L E D D A M E Q ↓ G V K D Y V E Q L G N A F G S G F T N Q
CCGTGACCTGTTGTGGTTGGAAGATGATGCAATGGAACAGGGAGTGAAAGATTACGTTGAGCAACTTGGTAATGCTTTTGGCTCAGGATTCACCAACCAG
3710 3730 3750 3770 3790
I C E Q V N L L K E S L V C Q D S I L E K S L K A L V K I I S A L V
ATATGCGAGCAGGTTAACCTCCTAAAAGAATCACTAGTAGGTCAGGACTCAATCTTGGAGAAGTCACTCAAAGCCCTAGTTAAGATCATCTCTGCCCTGG
3810 3830 3850 3870 3890
I V V R N H D D L I T V T A T L A L I G C T S S P W R W L K H K V
TGATTGTAGTAAGGAATCATGATGACCTGATCACAGTTACAGCTACACTCGCCCTTATTGGCTGCACCTCGTCTCCGTGGCGATGGCTTAAGCACAAGGT
3910 3930 3950 3970 3990
P2-B | P2-C
S Q Y Y G I P M A E R Q ↓ N N C W L K K F T E M T N A C K G M E W I
GTCCCAATATTACGGAATACCCATGGCTGAAGGCCAGAACAACGGGTGGCTAAAGAAATTTACAGAGATGACTAACGCATGCAAAGGGATGGAGTGGATA
4010 4030 4050 4070 4090
A V K I Q K F I E W L K V K I L P E V K E K H E F L S R L K Q L P L
GCCGTCAAGATTCAGAAATTTATAGAATGGCTCAAGGTTAAGATTTTGCCAGAAGTCAAGGAAAAGCATGAATTCCTAAGTAGACTCAAACAGCTCCCAC
4110 4130 4150 4170 4190
L E S Q I A T I E Q S A P S Q S D Q E Q L F S N V Q Y F A H Y C R
TCTTGGAGAGTCAGATTGCCACCATTGAACAAACTGCACCCTCTCAAAGTGACCAGGAACAGCTGTTCTCAAATGTCCAGTACTTCGCTCACTATTGCAG
4210 4230 4250 4270 4290
K Y A P L Y A A E A K R V F S L E K K F S N Y I Q F K S K C E I E
AAAGTACGCACCGCTCTACGCTGCAGAAGCCAAGAGGGTGTTTTCCCTTGAAAAAAAAATGAGCAATTACATACAGTTCAAGTCCAAATGCCGTATTGAA
4310 4330 4350 4370 4390
P V C L L L H G S P G A G K S V A T N L I G R S L A E K L N S S V Y
CCTGTATGCTTGCTTTTGCATGGTAGCCCAGGAGCCGGGAAGTCAGTTGCTACCAACTTAATTGCGCGGTCATTAGCTGAAAAGTCAAACAGTTCAGTGT
4410 4430 4450 4470 4490
S L P P D P D H F D G Y K G Q A V V I N D D L G G N P D G K D V S
ACTCCTTACCACCAGACCCAGACCATTTCGATGGCTACAAACAACAAGCCGTCGTAATTATGGACGATCTATGCCAAAACCCGGATGGCAAGGACGTGTC
4510 4530 4550 4570 4590
L F C Q N V S S V D F V P P M A A L E E K G I L F T S P F V L A S
TTTGTTCTGCCAAATGGTGTCTAGTGTAGACTTTGTACCACCAATGGCTGCACTGGAGGAAAAAGGTATCTTGTTCACCTCCCCTTTTGTCCTGGCCTCA
4610 4630 4650 4670 4690
T N A G S I N A P T V S D S R A L A R R F H F D K N I E V I S H Y S
ACCAATGCTGGGTCCATCAACCCGCCGACAGTCTCAGACAGCCGGGCTCTGGCAAGAAGGTTCCATTTTGACATGAAATATTGAAGTTATCTCCATGTACA
4710 4730 4750 4770 4790
VP1 | P2-A
Q N G K I N N P M S V K T C D F E C C P V N F K R C C P L V C G K
GCCAGAATGGCAAGATCAACATGCCCATGTCAGTCAAGACGTGTGATGAAGAGTGTTGCCCAGTCAATTTCAAGAGATGCTGCCCTCTAGTGTGTGGAAA
4810 4830 4850 4870 4890
A I C F I D R K T Q V E Y S L D E L V T E E F R E Y N H R H S V C
GCCTATCCAGTCTTATCGATAGAAAGACTCAAGTGAGGTACTCCCTAGATATGCTAGTCACGGAGATGTTTAGGGAATACAACCACAGGCACAGTGTCGGG
4910 4930 4950 4970 4990


P2-C | P3-A
P3-A | VPg
VPg | Protease (P3-C).
Protease | Polymerase (P3-D)

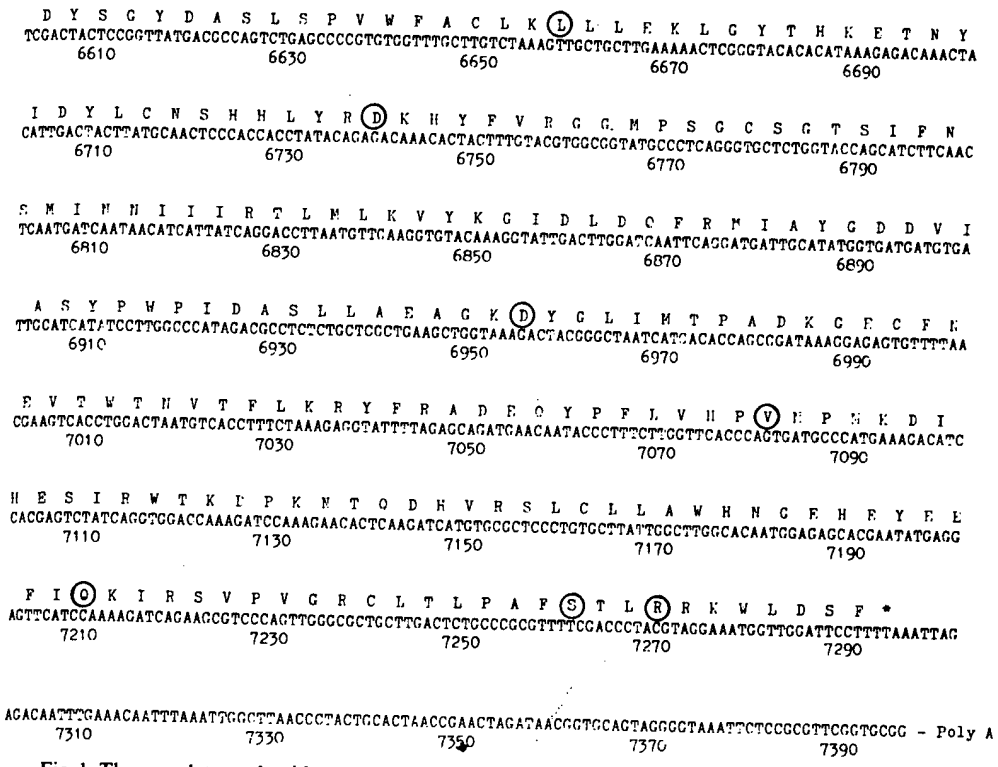



Fig. 1. The complete nucleotide sequence and predicted amino acid sequence of the polyprotein of CB4 (J.V.B. Benschoten). Amino acid differences from the previously sequenced CB3 (Nancy), are circled. Predicted polyprotein cleavage sites are arrowed.

Table 1. *Nucleotide sequence homologies between the non-coding regions of CB4 and four other picornaviruses**

| | CB4 : CB3 | CB4 : PV1 | CB4 : PV3 | CB4 : HRV14 |
|---|---|---|---|---|
| 5′ non-coding | 84·4 | 71·4 | 71·1 | 62·8 |
| 3′ non-coding | 93·8 | 59·1 | 59·1 | 45·2 |

* Sequence homologies are expressed as percentages. Details of the other virus sequences taken from Lindberg *et al.* (1987) for CB3, Kitamura *et al.* (1981) for PV1, Stanway *et al.* (1984*b*) for PV3 and Stanway *et al.* (1984*a*) for HRV14.

these correspond to those previously described as highly conserved between HRV14 and PV3 (Stanway *et al.*, 1984*a*). It has been suggested that these conserved sequences may be important for RNA secondary structure which is necessary for an, as yet, unspecified function in replication (Tracy *et al.*, 1985; Toyoda *et al.*, 1984; Newton *et al.*, 1985). Of interest in this respect is a region from nucleotides 10 to 34 which in the polioviruses has the potential to form a stable stem–loop secondary structure (Larsen *et al.*, 1981; Stanway *et al.*, 1983). In the corresponding region, CB4 is not well conserved in primary sequence but can form a similar stem–loop structure composed of exactly the same number of GC and AT base pairs. This stem–loop is likely to have functional importance in replication since it has been shown for PV2 that deletion of base 10 (which destabilizes the structure) leads to a mutant virus with a temperature-sensitive phenotype. This mutation appears to affect the rate of protein synthesis at both permissive and non-permissive temperatures (Racaniello & Meriam, 1987). Interestingly the corresponding regions in HRV14 and HRV2 do not appear to form such structures readily,

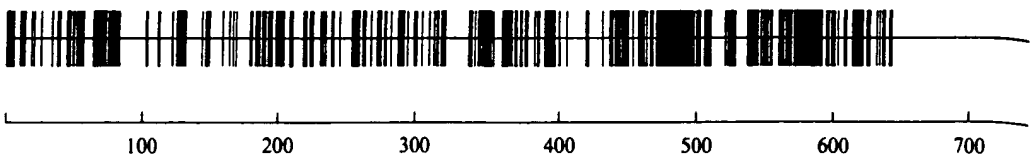



Fig. 2. Comparison between the 5′ non-coding regions of CB4 (J.V.B. Benschoten), CB3 (Nancy), PV1 (Mahoney), PV3 (P3/Leon/37) and HRV14. Vertical bars represent conserved nucleotides. Nucleotide numbers (Fig. 1) are indicated.

Table 2. *Proposed proteolytic cleavage sites in the polyproteins of five picornaviruses**

| Boundary | CB4 | CB3 | PV1† | PV3 | HRV14 |
|---|---|---|---|---|---|
| VP4/VP2 | N/S | N/S | N/S | N/S | N/S |
| VP2/VP3 | Q/G | Q/G | Q/G | Q/G | Q/G |
| VP3/VP1 | Q/G | Q/G | Q/G | Q/G | Q/T?<br>Q/T?<br>E/G? |
| VP1/P2-A | Y/G | Q/S?<br>I/R?<br>Y/R?<br>Q/N? | Y/G | Y/G | Y/G?<br>Y/G? |
| P2-A/P2-B | Q/G | Q/G | Q/G | Q/G | Q/G |
| P2-B/P2-C | Q/N | Y/G?<br>Q/N? | Q/G | Q/G | Q/A |
| P2-C/P3-A | Q/G | Q/G | Q/G | Q/G | Q/G |
| P3-A/VPg | Q/G | Q/G | Q/G | Q/G | Q/G |
| VPg/protease (P3-C) | Q/G | Q/G | Q/G | Q/G | Q/G |
| Protease/polymerase (P3-D) | Q/G | Q/G | Q/G | Q/G | Q/G |

* Details taken from Tracy *et al.* (1985) and Lindberg *et al.* (1987) for CB3, Stanway *et al.* (1984*b*) for PV3 and Stanway *et al.* (1984*a*) for HRV14.

† Cleavage sites in PV1 were determined by amino acid sequencing (Larsen *et al.*, 1982; Pallansch *et al.*, 1984).

suggesting that the function is not indispensable. The hundred or so nucleotides prior to the initiation of translation of the polyprotein, as in the polioviruses, are poorly conserved and it is unlikely that this region has any functional significance, although it has been suggested that it may play a role in the conservation of the length of the 5′ non-coding region (Toyoda *et al.*, 1984). This idea does not hold for the rhinoviruses, however, where the region appears to have been completely deleted.

### *Translated region*

Translation of the CB4 RNA probably initiates at nucleotide 744. The initiation codon at this position forms part of the sequence AAAAUGG, which is an almost optimal translation initiation sequence for eukaryotic ribosomes (Kozak, 1986). In this frame, there are no termination codons until nucleotide 7293. The region therefore can encode a polyprotein of 2183 amino acids, consistent with the known replication strategy of the picornaviruses (see Rueckert, 1985). The amino acid homology between the predicted proteins of CB4 and those of CB3, PV1, PV3 and HRV14 are shown in Table 3 and represented diagrammatically in Fig. 3. The homology with PV1, for which a detailed genetic map has been determined biochemically (Pallansch *et al.*, 1984; Kuhn & Wimmer, 1987) facilitates the identification of the sites in the CB4 polyprotein at which the virus-encoded proteases are likely to act. These are presented in Table 2 together with the assumed cleavage sites of CB3, PV3, HRV14, and the determined cleavage sites of PV1. With the exception of that at the P2-B/P2-C junction all of the cleavage sites in PV1 are conserved in CB4 and are therefore likely to be utilized. To identify the likely cleavage site between P2-B and P2-C, a comparison with CB3 is helpful. Lindberg *et al.* (1987)

Table 3. *Amino acid sequence homology between the proteins of CB4 and four other picornaviruses**

| Protein | CB4 : CB3 | CB4 : PV1 | CB4 : PV3 | CB4 : HRV14 |
|---|---|---|---|---|
| VP4 | 94·2 | 69·6 | 71·0 | 60·8 |
| VP2 | 80·5 | 56·0 | 56·3 | 54·6 |
| VP3 | 78·2 | 55·9 | 55·9 | 46·8 |
| VP1 | 71·2 | 47·3 | 44·6 | 35·7 |
| Total P1 | 77·9 | 54·4 | 54·1 | 47·9 |
| P2-A | 91·2 | 57·8 | 59·1 | 44·4 |
| P2-B | 97·9 | 50·5 | 51·5 | 55·7 |
| P2-C | 97·6 | 62·6 | 62·9 | 58·7 |
| P3-A | 92·1 | 47·1 | 50·6 | 48·2 |
| VPg | 90·9 | 77·3 | 72·7 | 40·9 |
| Protease (P3-C) | 95·0 | 60·7 | 60·6 | 52·7 |
| Polymerase (P3-D) | 95·2 | 74·0 | 73·9 | 65·9 |

* Sequence homologies are expressed as percentages. Details taken from Lindberg *et al.* (1987) for CB3, Kitamura *et al.* (1981) for PV1, Stanway *et al.* (1984*b*) for PV3 and Stanway *et al.* (1984*a*) for HRV14.

have proposed that in CB3 this cleavage occurs either at a tyrosine–glycine pair or a glutamine–asparagine pair which by alignment correspond to those in CB4 at nucleotide positions 4013 and 4037 respectively (Fig. 1, Table 2). In PV1 this cleavage occurs at a glutamine–glycine pair which by alignment corresponds exactly to the glutamine–asparagine pair in CB4. The amino acid sequence following this site is highly conserved between CB4, CB3 and PV1, whereas the amino acid sequence around the tyrosine–glycine pair (although conserved between CB4 and CB3) shows no homology to the corresponding region in the polioviruses. Moreover glutamine–asparagine has been shown by amino acid sequencing to be a cleavage site in HRV2 (Skern *et al.*, 1985). We conclude therefore that the glutamine–asparagine pair probably functions as the cleavage site for P2-B/P2-C in both CB3 and CB4.

Of interest also is the P1/P2 cleavage site occurring between a tyrosine and glycine residue in HRV14, and all serotypes of poliovirus (Table 2). The sequence comparisons indicate that this site is conserved in CB4 and is therefore likely to be used, but that it is not present in CB3 (Fig. 1, Table 2). Four alternative sites have been proposed for the corresponding cleavage in CB3 (Tracy *et al.*, 1985; Lindberg *et al.*, 1987) (Table 2). Unfortunately the two coxsackieviruses are very different in this region and the CB4 sequence does not therefore help to distinguish between the four possibilities for cleavage in CB3.

Cleavage of the poliovirus precursor polypeptide VP0 to give VP4 and VP2 occurs at an asparagine–serine amino acid pair and is thought to be an autocatalytic event (Hogle *et al.*, 1985). This site is also conserved at an equivalent position in the CB4 polyprotein (nucleotide position 950) and is likely therefore to function in a similar manner (Fig. 1).

Verification of these proposed polyprotein processing sites in CB4 would, of course, require N- and C-terminal protein sequence analysis of infected cell and virus structural polypeptides. However, the high degree of homology to the other enteroviruses in these regions of the polyprotein provides persuasive evidence that the cleavage sites identified in Table 2 for CB4 are correct. The sizes of the predicted CB4 proteins are summarized in Table 4.

### *Structural proteins*

The P1 region of the polyprotein forms the precursor to the four structural proteins VP1 to VP4. As expected, and in common with the rhinoviruses and most other enteroviruses, VP4 is the most conserved of the capsid proteins. In PV1 and HRV14, VP4 is not exposed on the outer surface of the virion (Hogle *et al.*, 1985; Rossmann *et al.*, 1985) and does not contribute to antigenic domains important in virus neutralization (Minor *et al.*, 1986). This protein is therefore unlikely to be subjected to immune selection pressure. In the three other structural proteins, VP1, VP2 and VP3, there are regions that are highly conserved, interspersed with regions of substantial divergence (Fig. 3). Regions of highest homology are those likely to be of importance in maintaining the stable secondary structure of the capsid proteins and these

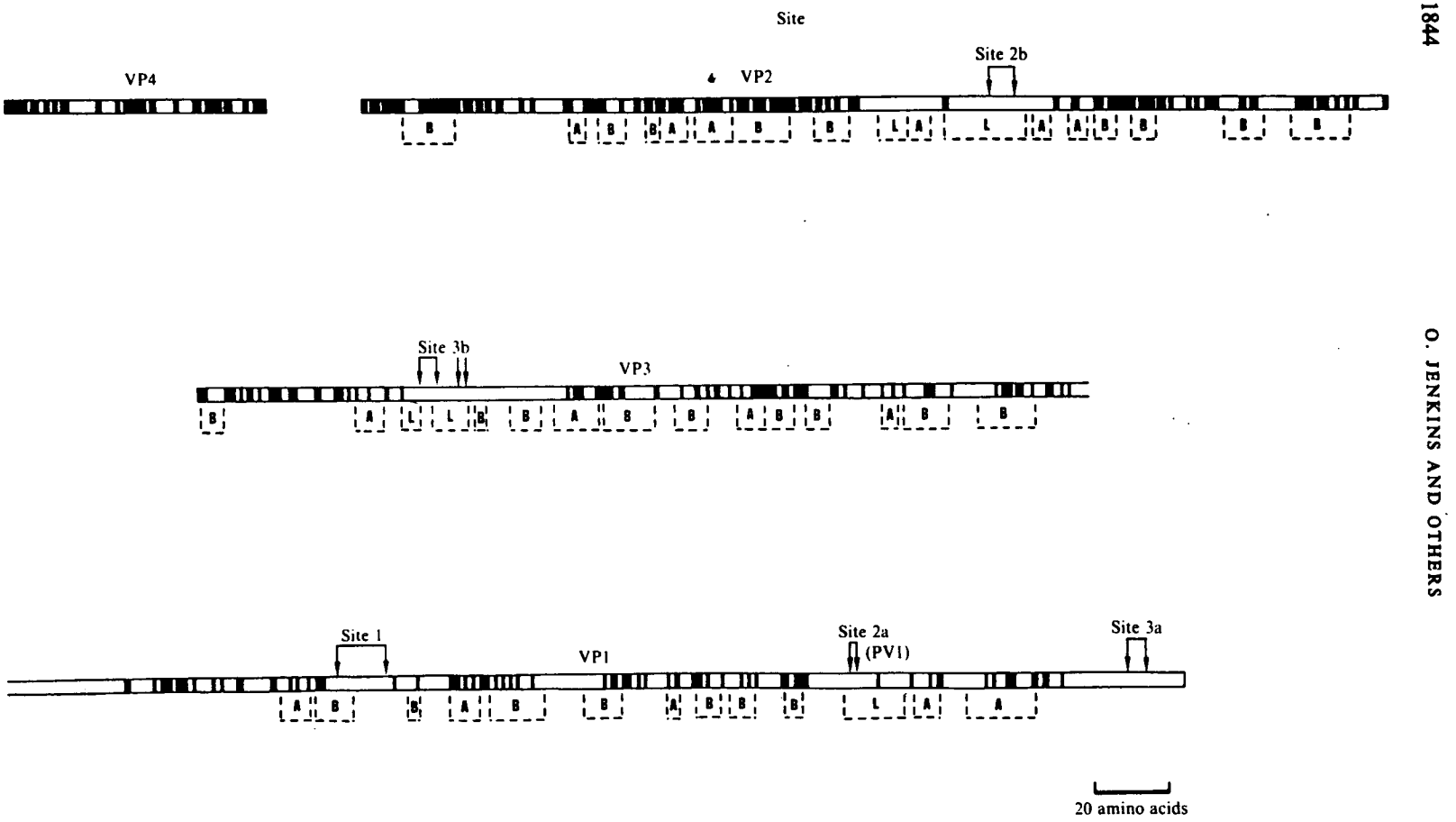



Fig. 3. Comparison between the capsid proteins of CB4 (J.V.B. Benschoten), CB3 (Nancy), PV1 (Mahoney), PV3 (P3/Leon/37) and HRV14. Conserved amino acids are represented by black boxes. Antigenic sites determined for PV3 are shown. Regions identified in the 3-D structure of PV1 as α-helices (A), β-sheets (B) and random coil or loop-out regions (L) are indicated.

Table 4. *CB4 proteins and their sizes**

| Protein | Number of amino acids | Mol. wt. ($\times 10^{-3}$) |
|---|---|---|
| VP4 | 69 | 7·49 |
| VP2 | 261 | 28·56 |
| VP3 | 238 | 26·43 |
| VP1 | 284 | 31·98 |
| P2-A | 147 | 16·22 |
| P2-B | 99 | 10·96 |
| P2-C | 329 | 37·27 |
| P3-A | 89 | 9·96 |
| VPg | 22 | 2·41 |
| Protease (P3-C) | 183 | 20·33 |
| Polymerase (P3-D) | 462 | 52·53 |
| Total polyprotein | 2183 | 243·96 |

* Based on the cleavage sites proposed in Table 2.

correspond, by alignment with PV1, to $\alpha$-helices and $\beta$-sheets in the three-dimensional structure (Hogle *et al.*, 1985). By contrast, the regions which show least homology correspond to the more flexible loop-out or random coil secondary structures, some of which are known to be antigenic determinants in polioviruses and rhinoviruses (Minor *et al.*, 1986; Rossmann *et al.*, 1985; Skern *et al.*, 1987; Sherry & Rueckert, 1985; Fig. 3). Thus it is likely that in CB4 the amino acids 76 to 87 in VP1, 158 to 164 in VP2 and 58 to 60 and 70 to 71 in VP3 are antigenically important. Although other amino acids are also likely to contribute to antigenicity, the imprecision of the alignment in these regions of random coil makes their identification less reliable.

### *Non-structural proteins*

The P2 region is the part of the polyprotein most highly conserved between the two coxsackieviruses B3 and B4. There are only two amino acid differences beween them in the P2-B protein and only eight differences in the P2-C protein (Fig. 1, Table 3). The functions of both of these proteins are unknown, but there is evidence that P2-C forms part of the membrane-bound replication complex (Takegami *et al.*, 1983). Mutations conferring guanidine resistance in PV1 have also been mapped to this protein (Pincus *et al.*, 1986). P2-A has been shown to function as a protease responsible for cleavage of the polyprotein at tyrosine–glycine cleavage sites (Toyoda *et al.*, 1986). As discussed above, the amino acid pair tyrosine–glycine is present at the P1/P2 junction in CB4, PV1, PV3 and HRV14 (Table 2) but is not present in CB3. It is therefore interesting to note that P2-A of CB4 is substantially more homologous to the P2-A of CB3 (91%) even though their substrates are different, than to those of the other viruses (less than 60%) in which the substrate cleavage site is the same.

The P3 region is processed to give P3-AB (which is subsequently processed to provide VPg), a protease (P3-C) and an RNA-dependent RNA polymerase (P3-D). The VPgs of coxsackieviruses B1, B3 and B5 have been compared at the amino acid level (Lindberg *et al.*, 1987). The VPg sequence of CB4 differs from those of CB1 and CB5 by only one amino acid, and differs from the CB3 sequence by two. These changes are conservative. As is the case in all enteroviruses both the protease (P3-C) and polymerase (P3-D) of coxsackieviruses are highly conserved (Fig. 1, Table 3), the polymerase being the most highly conserved protein of those specified by picornavirus genomes (Table 3; Argos *et al.*, 1984).

### *3′ non-coding region*

The translation of the polyprotein is terminated at position 7293 by the sequence UAA which is followed by a non-coding region of 100 nucleotides prior to the poly(A) tract (Fig. 1). This region is very highly conserved between CB4 and CB3 (94%, Table 1) and also between the three serotypes of polioviruses (98%, Toyoda *et al.*, 1984) but among the enteroviruses and rhinoviruses as a whole it is far less well conserved than the 5′ non-coding region. The function of this 3′ non-coding region in picornaviruses has not yet been determined, although it is likely to

(*a*)

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                1                                                    50
CB4     aattagagac aatttgaaac aatttaaatt ggcttAACCC TACtgca..c
CB3     gattagagac aa.ttgaaac aatttagatt ggcacAACCC TACtg..tgC
PV3     .......... .......... .......... ...gtAACCC TACctcagtC
Consens ---------- ---------- ---------- -----AACCC TAC------C

               51                                                   100
CB4     tAAccGaAct aGaTaAcggT gcaGTAGCGG TAAATTcTcC gcgttcggtG
CB3     tAAccGaAcc aGaTaAcggT acaGTAGCGG TAAATTcTcC gcattcggtG
PV3     gAAttGgAtt gGgTcAtacT gttGTAGCGG TAAATTtTtC tttaattcgG
Consens -A--G-A-- -G-T-A---T ---GTAGCGG TAAATT-T-C ---------G

               101
CB4     cGg
CB3     cG.
PV3     aG.
Consens -G-
```

(*b*)

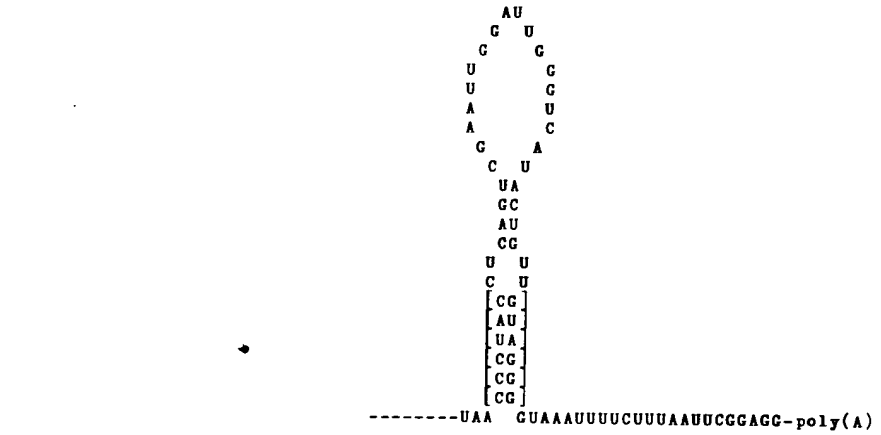


Fig. 4. (*a*) Alignment of the 3′ non-coding regions of CB4 (J.V.B. Benschoten), CB3 (Nancy) and PV3 (P3/Leon/37). Conserved nucleotides are shown in upper case. (*b*) Predicted RNA secondary stem–loop structure of this region. Conserved nucleotides underlined in (*a*) form part of the stem.

be involved in the control of genome replication (Fellner, 1979). The conserved blocks of nucleotides reported previously following comparisons of CB3, PV1 and swine vesicular disease virus (Stalhandske *et al.*, 1984) are also present in CB4 (Fig. 4). A number of different stem–loop secondary structures can be compiled for this region (Ryan, 1985) in which the conserved blocks of nucleotides form part of the stem. Occasionally sequence divergence within the stem is observed (e.g. in enterovirus 70; M. Ryan, unpublished), but base pairing is always maintained by compensatory mutations across the stem. It has been shown that the insertion of an eight nucleotide linker at position 7387 in the 3′ non-coding region of PV1 gives rise to a virus with a temperature-sensitive phenotype suggesting that the structure of the region has some essential function. The temperature-sensitive phenotype may result from destabilization of the secondary structure at the restrictive temperature (Sarnow *et al.*, 1986). It is interesting that HRV14 and HRV2 have much smaller 3′ non-coding regions than the enteroviruses. The conserved block of nucleotides closest to the poly(A) tract (Fig. 4) is deleted and it is therefore unlikely that the rhinoviruses are able to form similar secondary RNA structures. The significance of this observation is unclear, but it is interesting to note the parallel with the 5′ non-coding region as discussed above where the loop formed by nucleotides 10 to 34 is also missing in the rhinoviruses. These features may be consistent differences between the rhinoviruses and enteroviruses (Stanway *et al.*, 1984*a*).

It is clear that CB4 is very closely related to CB3 and to other members of the enterovirus genus. This high level of homology, particularly with the better studied members of the Picornaviridae such as PV1 and HRV14, allows a comprehensive interpretation of the sequence to be made. Thus, polyprotein cleavage signals can be confidently located and likely antigenic domains tentatively identified. These comparisons are useful in that they also indicate regions of sequence difference which must ultimately determine the characteristic biological properties which differ between picornaviruses. The possibility to manipulate these regions via site-directed mutagenesis of cloned cDNA provides an experimental approach to understanding the molecular basis of picornavirus diversity. Such experiments are in progress.

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VIRUS 00777

# Identification of candidate sequences that determine virulence in Coxsackievirus B4

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**Summary**

We have previously shown that a major determinant of virulence for coxsackievirus B4 mapped to the 5′ end of the viral genome. Comparison of the corresponding cDNA sequences of a virulent and a non-virulent virus has allowed the identification of candidate determinants of virulence in the 5′ untranslated region and the capsid proteins VP1, VP2 and VP4. Thirteen nucleotide substitutions were observed in a region spanning 3298 nucleotides. Four mutations were detected in the non-coding region. Of the remaining nine mutations, four were silent while five resulted in amino acid substitutions in VP1, VP2 and VP4. The amino acid substitutions in the virulent virus were analyzed in relation to the three-dimensional structures of the capsid proteins of poliovirus. Two substitutions mapped to the amino termini of VP1 and VP4. Of the two substitutions observed in VP2, one mapped to the large loop that connects beta strand E with the radial helix on the back surface of the eight-stranded antiparallel beta barrel while the other mapped to beta strand G. One amino acid substitution in VP1 mapped to the loop connecting beta strands D and E at a site close to a major determinant of attenuation in poliovirus type 2.

Coxsackievirus B4; Virulence; DNA sequence

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A great deal of information exists about the biochemical, biophysical and genetic characteristics of picornaviruses. However, the mechanisms by which these RNA viruses cause disease are poorly understood. Coxsackieviruses of the B group have been implicated in diseases such as pancreatitis, myocarditis, myositis and type I insulin-dependent diabetes mellitus (Grist et al., 1978; Melnick, 1985). Of the group B viruses, variants exist within a single serotype thereby contributing to the variability in the pathogenesis of coxsackievirus infections. We have previously described a variant of coxsackievirus B4 (CB4-V) that induces acute pancreatitis with concurrent hypoglycemia in mice (Ramsingh et al., 1989). The prototypical JVB strain (CB4-P), at similar titers, was non-virulent since infected mice did not develop disease.

A powerful tool in the investigation of the genetic basis of virulence of picornaviruses has been the use of recombinant, chimeric viruses constructed from infectious cDNA clones of virulent and non-virulent viruses (Kohara et al., 1988; Murray et al., 1988). Using this approach, we showed that, for coxsackievirus B4, a major determinant of virulence maps to the 5′ end of the genome, which encompasses the 5′ untranslated region (UTR) and the P1 region, which encodes the four capsid proteins (Ramsingh et al., 1990). Attenuation determinants for poliovirus have also been mapped to this region. Sequence analyses have identified two such determinants in the type 3 vaccine strain, P3/Sabin, and include a uridine at position 472 in the 5′ UTR and a phenylalanine at amino acid 91 of the capsid protein VP3 (Westrop et al., 1987; Minor et al., 1989). Similar studies using the type 1 vaccine strain have identified a strong attenuation determinant in the 5′ UTR, which corresponds to a guanine at position 480, although additional determinants are scattered throughout the genome (Kohara et al., 1985; Nomoto et al., 1987). Recent studies have identified two major determinants of attenuation in type 2 poliovirus (Ren et al., 1991; Equestre et al., 1991). These map to nucleotide 481 in the 5′ UTR and amino acid position 143 of the capsid protein VP1. For another picornavirus, Theiler's murine encephalomyelitis virus (TMEV), amino acid 101 of VP1 has been identified as an important determinant of neurovirulence (Zurbriggen et al., 1991).

In this report, comparison of the sequence data for the 5′ UTR and the P1 regions of a virulent and a non-virulent coxsackievirus B4 has allowed the identification of candidate determinants of virulence in the 5′ UTR and the capsid proteins VP1, VP2 and VP4. The capsid protein VP3 did not appear to contribute to the virulent phenotype. In addition, one amino acid substitution in VP1 mapped to a region very close to a major determinant of attenuation in poliovirus type 2.

The prototypical, non-virulent coxsackievirus B4 (strain JVB), herein designated CB4-P, was kindly provided by R. Deibel (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY). The origin and passage history of a virulent, pancreatropic variant of coxsackievirus B4 (CB4-V) has been previously described (Ramsingh et al., 1989). Viruses were grown in LLC-MK2(D) cells and viral infectivity was determined using a plaque assay or a microtiter $TCID_{50}$ assay (Minor, 1985). Using RNA extracted from purified preparations of either CB4-P or CB4-V, cDNA libraries were prepared as previ-

ously described (Ramsingh et al., 1990). This procedure generated several clones that spanned 95% of the viral genome. To clone the extreme 5′ end of the viral RNA, the technique of polymerase chain reaction (PCR) (Saiki et al., 1986) was used as previously described (Ramsingh et al., 1990). Briefly, oligonucleotide primers derived from the sequence of Jenkins et al. (1987) were used to amplify the 3′ end of the cDNA product that was synthesized by reverse transcription of viral RNA. The cDNA corresponding to bases 1 to 1153 of the viral RNA was amplified. An *Xba*I site was added to the 5′-end primer while an inherent *Eco*RI site was used for the 3′-end primer. The amplified products were subcloned into the phagemid pBSKSM13 + (Stratagene). The sequences of the cDNA inserts were determined by the dideoxy chain-termination method (Sanger et al., 1977) using coxsackievirus-specific primers and Sequenase (U.S. Biochemical). The cDNA inserts were sequenced in both the forward and reverse orientations. Sequence data for the 5′ UTR and the P1 regions of both CB4-V and CB4-P were assembled and analyzed using the Wisconsin GCG Sequence Analysis Software Package (Devereux et al., 1984). Sequence differences between CB4-V and CB4-P were confirmed by RNA sequencing. RNA was sequenced directly by a modification of the dideoxy chain-termination method (Sanger et al., 1977) using virus-specific primers and reverse transcriptase (Life Sciences, St. Petersburg, FL).

Comparison of the cDNA sequence of the 5′ UTR and the P1 region of CB4-V with that of CB4-P revealed a total of 13 mutations, all of which consisted of nucleotide substitutions (Fig. 1). Four substitutions were detected in the 5′ UTR at positions 171, 638, 668 and 683. Of the nine mutations in the coding region for the capsid proteins, four were silent (Table 1). The remaining five mutations occurred in all three codon positions and resulted in amino acid substitutions in the VP1, VP2 and VP4 capsid proteins. Although a point mutation at nucleotide position 2240 in VP3 was noted, this change was silent. The VP1 and VP2 capsid proteins each contained two mutations while the VP4 protein contained one amino acid substitution. In CB4-V, amino acids 20 ($Arg^{20}$) and 129 ($Thr^{129}$) of VP1 were different to that of CB4-P ($Ser^{20}$, $Met^{129}$). For VP2 of CB4-V, amino acids 135 ($Ala^{135}$) and 201 ($Ala^{201}$) were altered (CB4-P, $Thr^{135}$, $Val^{201}$). In VP4 of CB4-V, the mutation was at amino acid 16 ($Arg^{16}$) (CB4-P, $Ser^{16}$). Of the amino acid substitutions, $Ala^{201}$ of VP2 (CB4-V) represented a conservative change while the remaining substitutions resulted in alterations in hydrophobicity or charge. $Ala^{135}$ of VP2 (CB4-V) is more hydrophobic while $Thr^{129}$ of VP1 (CB4-V) is more hydrophilic than the corresponding residues in CB4-P. Two mutations, $Arg^{16}$ and $Arg^{20}$ at the amino termini of VP4 and VP1, respectively, resulted in charge differences.

Comparison of our sequence data for CB4-P (strain JVB) with those of another sequence of strain JVB by Jenkins et al. (1987), herein referred to as CB4*, revealed a total of nine nucleotide substitutions between these two viruses (Fig. 1). The mutations probably reflect the different passage histories of the two viruses. Four of the substitutions mapped to the 5′ UTR at nucleotide positions 136, 137, 546 and 668. Of the five remaining mutations, two were silent and three resulted in amino acid substitutions (Table 1). These three changes mapped to the capsid

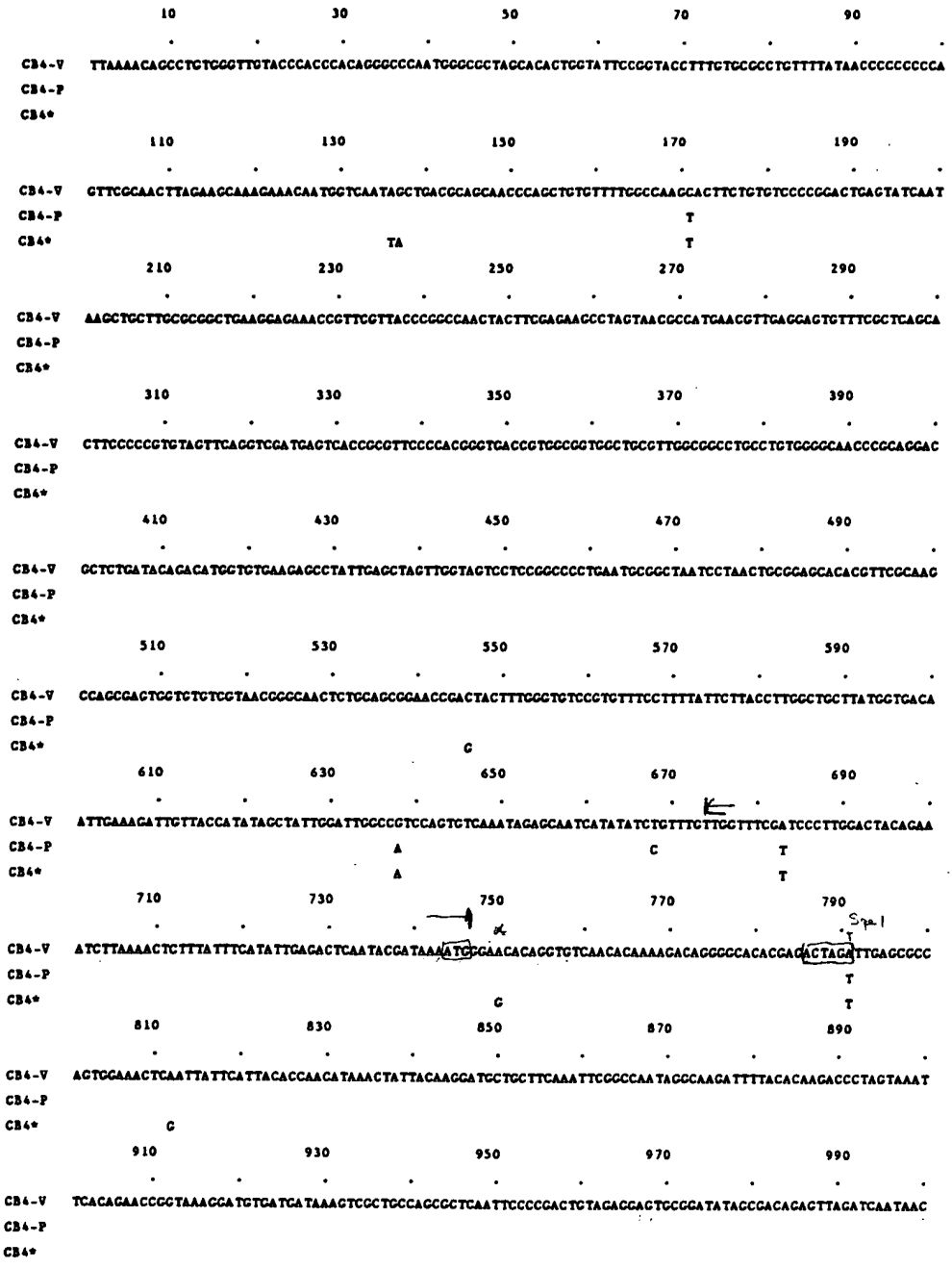


Fig. 1. (pp 284–287). Comparison of the cDNA sequences of the 5' untranslated region (nucleotides 1–743) and the P1 region (nucleotides 744–3298) of CB4-V, CB4-P and CB4* (Jenkins et al., 1987).

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                1010                1030                1050                1070                1090
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   ACTCGGGAACTCGACTATAACGACACAAGAGTGTGCAAACGTCGTGGTGGGGTATGGCGTCTGGCCCGATTATCTTAGCGACGAAGAGGCAACAGCCGAA
CB4-P
CB4*

                1110                1130                1150                1170                1190
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   GACCAACCCACCCAACCTCATGTGGCAACGTGTAGGTTTTACACGTTGAATTCAGTGAAATGGGACATGCAGTCAGCGGGGTGGTGGTGGAAGTTCCCAG
CB4-P   G
CB4*    G
                1210                1230                1250                1270                1290
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   ATGCATTGTCAGAAATGGGGCTCTTTGGGCAGAATATGCAGTATCACTACCTAGGCAGATCAGCGGTACAGAATTCATGTGCAATGCAACGCATCCAAATT
CB4-P                                       A
CB4*                                        A
                1310                1330                1350                1370                1390
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   CCACCAAGGTTGTCTGCTTGTGGTGTGTGTGCCTGAGGCTGAGATGGGATGTGCCAATGCAGAAAACGCACCCGCGTATGGTGATTTGTGTGGAGGAGAG
CB4-P                                             A
CB4*                                              A
                1410                1430                1450                1470                1490
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   ACAGCAAAGAGTTTCGAACAGAATGCAGCCACAGGTGAGACAGCTGTGCAGACGGCTGTGTGCAATCCGGGTATGGTGTCGGGGTTGGTAACTTCACTA
CB4-P
CB4*                                    A
                1510                1530                1550                1570                1590
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   TATACCCTCACCAATGGATTAATTTAAGAACAAACAATAGTGCCACCATAGCGATGCCATACATTAATAGCGTCCCAATGGACAACATGTTCAGGCATAA
CB4-P                                             T
CB4*                                              T
                1610                1630                1650                1670                1690
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   TAACTTTACATTAATGATAATACCCTTTGCACCGTTGGACTACGTTACGGGACGGTCCTCTTACATCCCTATCACAGTGACAGTTGCCCCTATGAGCGCT
CB4-P
CB4*
                1710                1730                1750                1770                1790
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   GAGTACAATGGTTTGCGTCTAGCTGGTCATCAAGGCTTACCAACTATGCTTACACCAGGCAGCACGCAGTTTTTGACGTCAGATGATTTTCAATCACCAT
CB4-P
CB4*
                1810                1830                1850                1870                1890
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   CAGCTATGCCACAGTTTGATGTGACCCCAGAGATGAACATTCCAGGGCAAGTGACGAACCTGATGGAAATTGCGGAAGTTGATTCTGTGGTACCAATCAA
CB4-P
CB4*
                1910                1930                1950                1970                1990
                 .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .    .
CB4-V   TAACTTGAAAGCTAATCTGATGACGATGGAGGCTTACCGGGTGCAGGTTAGGTCCACTGACGAGATGGGAGGACAGATATTTGGCTTCCCCTTACAGCCA
CB4-P
CB4*
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Fig. 1 (continued).

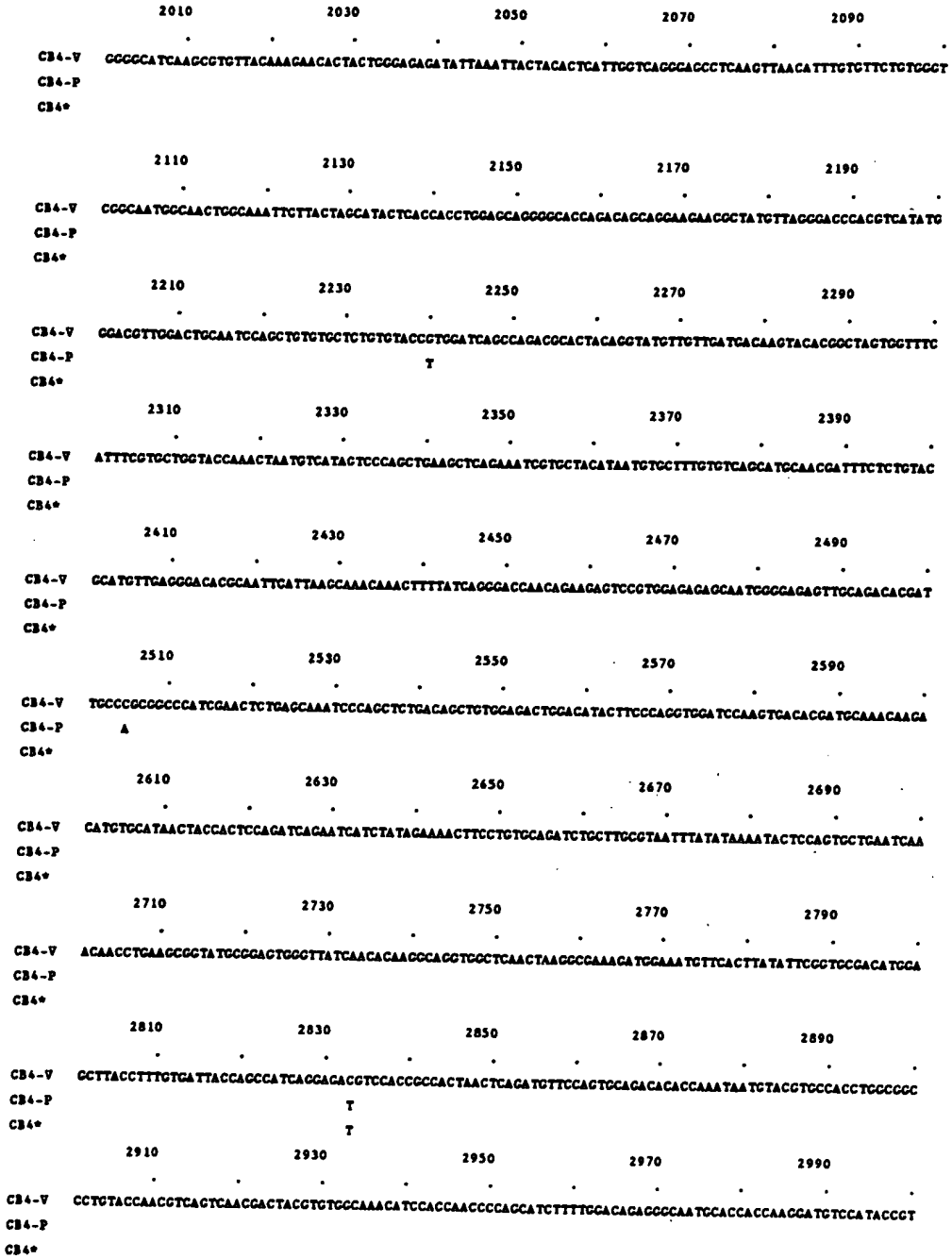


Fig. 1 (continued).

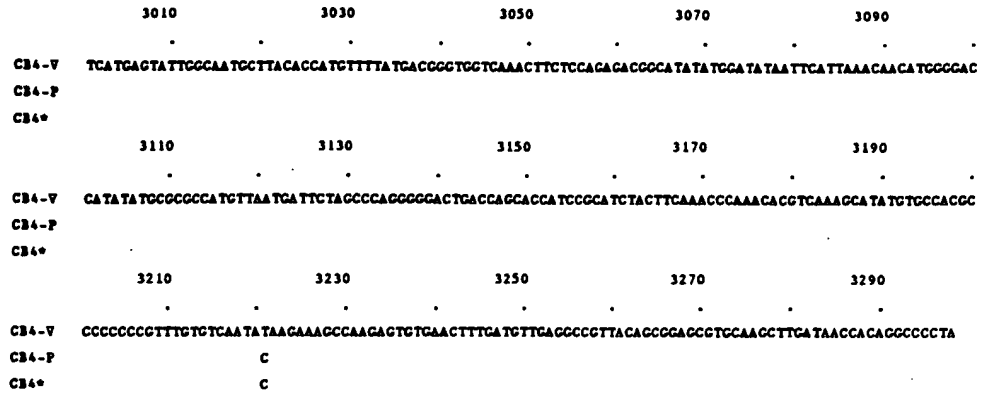


Fig. 1 (continued).

proteins VP4, VP2 and VP1. Again, although a point mutation was observed in VP3 at nucleotide position 2240, this change was silent. CB4-P contained $Thr^{3}$ (VP4), $Glu^{163}$ (VP2) and $Ser^{20}$ (VP1) while the JVB strain sequenced by Jenkins et al. (1987) had $Ala^{3}$ (VP4), $Lys^{163}$ (VP2) and $Arg^{20}$ (VP1).

TABLE 1

Summary of sequence data for the 5'UTR and the P1 regions of CB4-V, CB4-P and CB4*

| Sequenced nucleotide | Genome region | Nucleotide | | | | Amino acid | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Position | CB4-P | CB4-V | CB4* | Position | CB4-P | CB4-V | CB4* |
| 1-743 | 5'UTR | 136 | A | A | T | | | | |
| | | 137 | G | G | A | | | | |
| | | 171 [a] | T | C | T | | | | |
| | | 546 | C | C | G | | | | |
| | | 638 [a] | A | G | A | | | | |
| | | 668 [b] | C | T | T | | | | |
| | | 683 [a] | T | A | T | | | | |
| 744-949 | VP4 | 750 | A | A | G | 3 | Thr | Thr | Ala |
| | | 791 [a] | T | A | T | 16 | Ser | Arg | Ser |
| | | 812 | A | A | G | 23 | Ser | Ser | Ser |
| 950-1732 | VP2 | 1106 | G | A | G | 52 | Gly | Gly | Gly |
| | | 1241 | A | G | A | 97 | Gly | Gly | Gly |
| | | 1353 [a] | A | G | A | 135 | Thr | Ala | Thr |
| | | 1437 | G | G | A | 163 | Glu | Glu | Lys |
| | | 1552 [a] | T | C | T | 201 | Val | Ala | Val |
| 1733-2446 | VP3 | 2240 | T | G | G | 169 | Pro | Pro | Pro |
| 2447-3298 | VP1 | 2505 [b] | A | C | C | 20 | Ser | Arg | Arg |
| | | 2833 [a] | T | C | T | 129 | Met | Thr | Met |
| | | 3221 | C | T | C | 258 | Tyr | Tyr | Tyr |

[a] Potential determinant of virulence.

[b] Common nucleotide between CB4-V and CB4*.

**A**

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                1                                                  *      50
CB4-V VP1   .......GPT EESVERAMGR VADT...... IARGPSNSEQ IPALTAVETG
Polio VP1   GIGDMIEGAV EGITKNALVP PTSTNSLPGH KPSGPAHSKE IPALTAVETG
               51                                                     100
CB4-V VP1   HTSQVDPSDT MQTRHVHNYH SRSESSIENF LCRSACV..I YIKYSSAESN
Polio VP1   ATNPLVPSDT VQTRHVIQRR TRSESTVESF FARGACVAII EVDNDAPTKR
              101                                                  *  150
CB4-V VP1   NLKRYAEWVI NTRQVAQLRR KMEMFTYIRC DMELTFVITS HQETSTATNS
Polio VP1   ASRLFSVWKI TYKDTVQLRR KLEFFTYSRF DMEFTFVVTS ..NYIDANNG
              151                                                  *  200
CB4-V VP1   DVPVQTHQIM YVPPGGPVPT SVNDYVWQTS TNPSIFWTEG NAPPRMSIPF
Polio VP1   HALNQVYQIM YIPPGAPIPG KWNDYTWQTS SNPSVFYTYG APPARISVPY
              201                                                    250
CB4-V VP1   MSIGNAYTMF YDGWSNFS.. ......RDGI YGYNSLNNMG TIYARHVNDS
Polio VP1   VGIANAYSHF YDGFAKVPLA GQASTEGDSL YGAASLNDFG SLAVRVVNDH
              251                                                    300
CB4-V VP1   SPGGLTSTIR IYFKPKHVKA YVPRPPRLCQ YKKAKSVNFD VEAVTAERAS
Polio VP1   NPTRLTSKIR VYMKPKHVRV WCPRPPRAVP Y.FGPGVDYK .DGLTPLPEK
              301
CB4-V VP1   LITTGPY
Polio VP1   GLTTY..
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                1                                                      50
CB4-V VP2   SPTVEECGYS DRVRSITLGN STITTQECAN VVVGYGVWPD YLSDEEATAE
Polio VP2   SPNIEACGYS DRVMQLTLGN STITTQEAAN SVVAYGRWPE YIRDTEANPV
               51                                                     100
CB4-V VP2   DQPTQPDVAT CRFYTLNSVK WEMQSAGWWW KFPDALSEMG LFGQNMQYHY
Polio VP2   DQPTEPDVAA CRFYTLDTVT WRKESRGWWW KLPDALKDMG LFGQNMFYHY
              101                                                  *  150
CB4-V VP2   LGRSGYTIHV QCNASKFHQG CLLVVCVPEA EMG.......  ...CANAENA
Polio VP2   LGRAGYTVHV QCNAFKFHQG ALGVFAVPEM CLAGDSTTHM FTKYENANPG
              151                                                    200
CB4-V VP2   PAYGDLCGGE TAKSFEQNAA TGETAVQTAV CNAGMGVGVG NLTIYPHQWI
Polio VP2   EKGGEFKGSF T...LDTNAT NPARNFCPVD YLFGSGVLVG NAFVYPHQII
              201          *                                         250
CB4-V VP2   NLRTNNSATI AMPYINSVPM DNMFRHNNFT LMIIPFAPLD YVTGASSYIP
Polio VP2   NLRTNNCATL VLPYVNSLSI DSMTKHNNWG IAILPLAPLD FVTESSTEIP
              251                                273
CB4-V VP2   ITVTVAPMSA EYNGLRLAGH Q..
Polio VP2   ITLTIAPMCC EFNGLRNITV PRT
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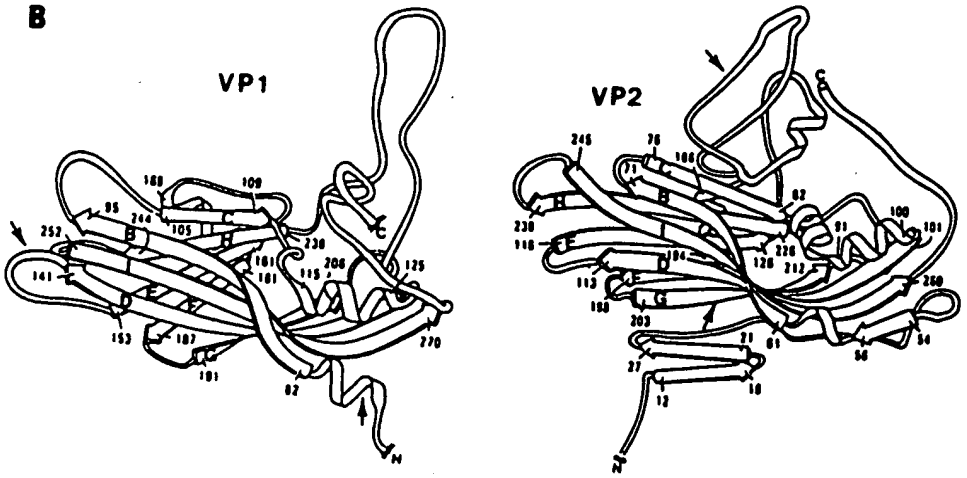
**B**



There is a high degree of similarity between the predicted amino acid sequences of coxsackieviruses and polioviruses. Comparison of the amino acid sequences of VP1 and VP2 of CB4-V to that of poliovirus type 2 (P712) (Toyoda et al., 1984) revealed sequence identities of 43.6% and 54.9%, respectively. Therefore, the amino acid changes observed in CB4-V were analyzed in relation to the three-dimensional structures of the capsid proteins of poliovirus. Alignment of the amino acid sequences of VP1 and VP2 of CB4-V with the corresponding capsid proteins of poliovirus type 2 (P712) was accomplished with the GCG program PILEUP (Fig. 2A). Using data from the three-dimensional structure of poliovirus type 1 (Mahoney strain) (Hogle et al., 1985) (permission kindly granted by J. Hogle and the AAAS, copyright 19 by the AAAS), this alignment allowed an approximation of the region of the molecule where the substituted amino acids in CB4-V mapped (Fig. 2B). By this comparison, $Thr^{129}$ of VP1 would map to the loop connecting beta strands D and E while $Arg^{20}$ would map to the amino terminus. $Ala^{135}$ of VP2 would map to the large loop that connects beta strand E with the radial helix on the back surface of the eight-stranded antiparallel beta barrel while $Ala^{201}$ would map to beta strand G. From this alignment, we can predict that $Thr^{129}$ (VP1) and $Ala^{135}$ (VP2) would be on the surface of the virion while $Arg^{20}$ (VP1), $Ala^{201}$ (VP2) and $Arg^{16}$ (VP4) would be expected to reside in the interior of the virion.

Comparison of the cDNA sequences of a virulent coxsackievirus B4 (CB4-V) and a non-virulent virus (CB4-P) revealed a total of thirteen point mutations. Four mutations occurred in the 5′ UTR while nine changes were observed in the P1 region. Of the nine mutations, five resulted in amino acid substitutions while four were silent. Comparison of the cDNA sequences of the 5′ UTR and the P1 regions of the two JVB strains of coxsackievirus B4 (CB4-P and CB4*) revealed nine nucleotide substitutions, which probably reflect the different passage histories of the two viruses. Four nucleotide changes were observed in the 5′ UTR while five substitutions were seen in the P1 region. Of the five changes, two were silent while three resulted in amino acid substitutions. This analysis suggests that CB4-V and CB4-P are just as similar as the two JVB strains, CB4-P and CB4*. All of the amino acid differences observed in the P1 region of the various strains occurred in the capsid proteins VP1, VP2 and VP4.

Of the thirteen point mutations observed between CB4-V and CB4-P, three (nucleotide positions 668, 2240 and 2505) were shared between the two JVB strains (Table 1). Since the point mutation at position 2240 is silent, these data suggest that the candidate determinants of virulence for CB4-V may be narrowed further to include three nucleotides in the 5′ UTR (171, 638, 683), $Arg^{16}$ of VP4, $Ala^{135}$

Fig. 2. Alignment of the capsid proteins VP1 and VP2 of CB4-V to those of poliovirus. (A) Alignment of the amino acid sequences of VP1 and VP2 of CB4-V with the corresponding capsid proteins of poliovirus type 2 (Toyoda et al., 1984) using the GCG program PILEUP. (B) Ribbon diagrams of VP1 and VP2 of poliovirus type 1 (Hogle et al., 1985) (permission granted by J. Hogle and the AAAS, copyright 19 by the AAAS). *, potential determinant of virulence in CB4-V; arrowhead, determinant of attenuation in poliovirus type 2 (Ren et al., 1991; Equestre et al., 1991); arrows, approximation of sites that potentially contribute to virulence in CB4-V.

and $Ala^{201}$ of VP2 and $Thr^{129}$ of VP1. The capsid protein VP3 apparently does not contribute to virulence in coxsackievirus B4 in our system.

Alignment of the amino acid sequences of VP1 and VP2 of CB4-V with that of poliovirus type 2 (P712) allowed an approximation of the regions of the molecules where the mutations in CB4-V occurred. Interestingly, amino acid 129 ($Thr^{129}$) of VP1 of CB4-V aligned with amino acid 142 ($Tyr^{142}$) of VP1 of poliovirus. The alignment positions $Thr^{129}$ on the loop connecting beta strands D and E. In relation to the canyon structure (Rossmann, 1989), the alignment of $Thr^{129}$ places this residue on the rim of the canyon. Recently, it has been shown that amino acid 143 in VP1 of poliovirus type 2 is a major determinant of attenuation (Ren et al., 1991; Equestre et al., 1991). Thus, one of the mutations observed in CB4-V occurred at a position very close to this site in poliovirus. The DE loop is exposed on the external surface of the virion and, in poliovirus, influences both host range and immunogenicity (Ren et al., 1991; Wieggers et al., 1989).

Two mutations in CB4-V, $Arg^{20}$ and $Arg^{16}$ occurred at the amino termini of VP1 and VP4, respectively. These two mutations are expected to be in the interior of the virion. The gain of positive charges that they introduce may affect the interactions of VP1 and VP4 with the negatively-charged viral genomic RNA. For VP2 of CB4-V, a mutation ($Ala^{201}$) was observed in beta strand G, which is also in the interior of the virion. However, $Ala^{201}$ represents a conservative amino acid change and is not expected to affect virulence. The second, non-silent mutation in VP2 of CB4-V ($Ala^{135}$) occurred in the large loop that connects beta strand E with the radial helix on the back surface of the barrel and again, this region is predicted to be exposed on the surface of the virion, on the rim of the canyon. Of the three amino acid substitutions observed between the two JVB strains, one also maps to the large loop in VP2 while the remaining two map towards the amino termini of VP1 and VP4.

Of the four point mutations in the UTR, which comprises a total of 743 nucleotides, three were clustered towards the 3′ end of this region (positions 638, 668 and 683) while one mutation occurred towards the 5′ end (position 171). Attenuation determinants have been mapped to the 5′ UTR in all three serotypes of poliovirus (Equestre et al., 1991; Kohara et al., 1985; Nomoto et al., 1987; Ren et al., 1991; Westrop et al., 1987). These determinants cluster in the middle of the 5′ UTR and include nucleotide positions 480, 481 and 472 of poliovirus types 1, 2 and 3 respectively. Thus, the four point mutations in the 5′ UTR of CB4-V occur at sites different to those observed for polioviruses.

For coxsackievirus B4, candidate determinants of virulence have been localized to the 5′ UTR and the capsid proteins VP1, VP2 and VP4. For the polioviruses, determinants of attenuation have been mapped to the 5′ UTR and the capsid proteins VP1 and VP3 (Equestre et al., 1991; Kohara et al., 1985; Minor et al., 1989; Nomoto et al., 1987; Ren et al., 1991; Westrop et al., 1987). Of the nine potential sites in CB4-V, one ($Thr^{129}$) maps to the DE loop of VP1, at a position close to a major determinant of attenuation in poliovirus type 2 ($Ile^{143}$). The remaining eight sites in CB4-V do not coincide with any other determinants in poliovirus. Should $Thr^{129}$ of VP1 be a determinant of virulence in CB4-V, then the

molecular mechanisms underlying virulence in coxsackieviruses and polioviruses may share some common features.

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# Severity of disease induced by a pancreatropic Coxsackie B4 virus correlates with the H-2K$^q$ locus of the major histocompatibility complex

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**Summary**

Coxsackie B viruses are known etiological agents of pancreatic diseases, including diabetes. The pathogenesis of these infections is influenced by both host and viral factors. In this report, we examined whether the outcome of Coxsackie B4 virus infection is dependent on the genes within the major histocompatibility complex (MHC). We generated a pancreatic variant, CB4-V and established an animal model system of pancreatitis with concurrent hypoglycemia in mice. Infection of various B10 H-2 congenic strains of mice revealed that the development of hypoglycemia with accompanying pancreatitis was independent of the MHC haplotype. However, the severity of the disease as monitored by the extent and duration of hypoglycemia and by mortality rate was found to be associated with the H-2 haplotype, specifically the H–2K$^q$ locus. Pancreatic damage induced by CB4-V appeared to be both immune-mediated and viral-mediated. Histological examination of pancreatic tissue from infected B10 H-2 congenic mice revealed an association between acute destruction of the exocrine pancreas and lymphocytic infiltration. This infiltration may correlate with immune-mediated destruction of the infected pancreatic tissue. Since preferential replication of CB4-V was not observed in the most susceptible B10 mouse strain, direct viral destruction may not be the major mechanism of pancreatic injury.

Coxsackie B4; Major histocompatibility complex

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## Introduction

The association between Coxsackie viruses of the B group and pancreatic diseases including diabetes has been recognized for many years (Dalldorf and Gifford, 1952; Gamble et al., 1969; Pappenheimer et al., 1951). Initial studies revealed that several Coxsackie B viruses caused extensive destruction of pancreatic acinar tissue in mice without identifiable changes in the cells of the islets of Langerhans (Dalldorf and Gifford, 1952; Pappenheimer et al., 1951). Ross et al. (1974) correlated pathological changes produced in the mouse pancreas by Coxsackie B (CB) viruses with serum amylase and glucose concentrations. They found that, although CB4 infections were less severe than those of CB1, CB3 and CB5, CB4 induced an acinar pancreatitis with a transient increase in serum amylase and a transient decrease in glucose levels. These results contrasted with those of Lansdowne (1976) who showed that the pathogenicity of CB4 closely resembled that seen in animals infected with CB3 and was more severe than that associated with CB1 and CB5 infections. In neither study were visible changes in the islets of Langerhans observed. Subsequent studies by Yoon et al. (1978a, b) and Tóniolo et al. (1982) showed that Coxsackie B viruses could infect pancreatic $\beta$ cells. Furthermore, inoculation of mice with CB4 virus that had been passaged in mouse $\beta$ cell cultures resulted in the development of diabetes. Yoon et al. (1978b) suggested that most strains of Coxsackie B virus are minimally $\beta$ cell-tropic and that if $\beta$ cells are damaged, the number is usually insufficient to produce detectable alterations in glucose metabolism. However, the tropism of these viruses for insulin-producing cells can be increased by serial passage in $\beta$ cell cultures. It appears that diabetogenic variants of Coxsackie B virus exist in nature since Yoon et al. (1979) isolated a diabetogenic variant of CB4 from a patient with diabetic ketoacidosis. Thus, the different diseases produced by Coxsackie B viruses may be due to variants with different tropisms and biologic properties within a mixed viral population.

Genetic factors appear to play a role in determining susceptibility to viral infections. Early studies showed that development of encephalomyocarditis (EMC) virus-induced diabetes in various inbred strains of mice is genetically determined and that susceptibility is inherited as a recessive trait (Ross et al., 1976; Yoon and Notkins, 1976). Analysis of the F2 data suggested that more than one gene was involved (Ross et al., 1976). Later studies from backcross data implied that susceptibility to EMC-induced diabetes was primarily controlled by a single locus involving two or more alleles (Onodera et al., 1978). Further work has suggested that genetically determined differences in viral receptors on the surface of pancreatic $\beta$ cells may be one of the factors controlling susceptibility to EMC-induced diabetes. Susceptibility to CB4-induced diabetes is also genetically determined (Yoon et al., 1978b). As is the case in EMC-induced diabetes, only certain inbred strains of mice develop diabetes when injected with CB4 virus. Most of the strains susceptible to EMC-induced diabetes are also susceptible to CB4-induced diabetes. However, the role of the major histocompatibility complex (MHC) in EMC or CB4 viral infections has not been defined. The present work was undertaken to examine the pathogenesis of CB4 virus infections and to specifically address whether

susceptibility to a pancreatropic variant of CB4 virus was influenced by the genes within the MHC. Since CB4 virus normally infects the acinar cells of the exocrine pancreas, these studies were carried out using a pancreatic variant, CB4-V, which caused acinar pancreatitis with concurrent hypoglycemia in mice.

## Materials and Methods

### *Virus and cells*

Coxsackie B4 (JVB) was kindly provided by R. Deibel (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY). A Coxsackie B4 isolate that was originally diabetogenic and that was subsequently grown in LLC-MK2(D) cells was provided by J.W. Yoon (University of Calgary, Alberta, Canada). Large-scale stock preparations of these viruses were prepared in either HeLa or LLC-MK2(D) cells. Viral infectivity was determined using a microtiter $TCID_{50}$ assay (Minor, 1985).

### *Mice*

SJL mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Breeders of strains B10.Q, B10.S(12 R) and B10.T(6R) and B10.AQR and B10.AKM were provided by J. Stimpfling (The McLaughlin Research Institute, Great Falls, Montana) and K. Frederick (Washington University, St. Louis, MO), respectively. All mice used in these experiments were 4–6-week-old males and were maintained 3 per cage. Mice were allowed to eat and drink ad libitum. Mice were injected intraperitoneally (IP) with virus diluted in phosphate-buffered saline (PBS). Control mice were injected IP with PBS. All injected animals were monitored daily. Animals found to be moribund were euthanized immediately by $CO_2$ overdose and counted as fatalities. All animal procedures were in accord with Department of Health, Education, and Welfare (DHEW) Publ. No. (NIH) 86–23, "Guide for the Care and Use of Laboratory Animals", and had been reviewed and approved by the Wadsworth Center Animal Welfare Committee.

### *Glucose assay*

Non-fasted mice were bled from the tail vein and serum glucose concentrations were determined by the glucose oxidase method (Raabo and Terkildsen, 1960). For glucose tolerance tests, blood samples were collected before and 60 min after intraperitoneal administration of 2 mg of D-glucose per gram body weight. Mice were fasted during the 60 min glucose challenge.

### *Histopathology*

At autopsy, the pancreas was removed aseptically and rinsed in sterile PBS. One half of the pancreas was fixed in phosphate-buffered formalin and sections were

stained by hematoxylin and eosin. The other half was processed for the presence of infectious virus. These mice were euthanized by ether overdose.

*Extraction of virus from various organs*

Pancreas, heart, kidney and spleen were removed aseptically and rinsed in sterile PBS. After mincing in PBS, cells were broken by homogenization in a Dounce tissue grinder. Cell-associated virus was released after 3 cycles of freezing and thawing. After a clarifying spin at $5000 \times g$ for 10 min to remove cellular debris, the supernate was collected and filtered through a 0.2 $\mu$M filter. Homogenates were tested for viral infectivity using a microtiter $TCID_{50}$ assay (Minor, 1985).

## Results

*Coxsackie B4 virus variant induces severe and prolonged hypoglycemia in SJL mice*

The Coxsackie B4 virus, obtained from Dr. Yoon (University of Calgary, Alberta, Canada), had initially been passaged 15 times in human pancreatic $\beta$ cells and was diabetogenic in susceptible mouse strains. Subsequent passages had been carried out in LLC-MK2(D) cells. To test whether this virus had retained its diabetogenic potential, each of twenty 4–6-week-old male SJL mice was injected intraperitoneally (IP) with $10^{6.3}$ $TCID_{50}$ of virus and serum glucose concentrations were monitored at various times post-infection (PI). These animals remained normoglycemic during the 8 weeks PI (data not shown) suggesting that the diabetogenic variants within this mixed viral population were lost by passaging in LLC-MK2(D) cells. To enhance the pancreatropic nature of this virus, it was then passaged in mice (via pancreatic homogenates) (Ross et al., 1974) a total of 5 times. The resulting virus has been designated CB4-variant (CB4-V).

To determine whether CB4-V differed from the prototypical CB4 (JVB) herein designated (CB4-P) virus, SJL mice were injected IP with $10^{4.3}$ $TCID_{50}$ of virus or 0.2 ml PBS and serum glucose concentrations were determined at various times PI (Fig. 1). Mice infected with CB4-P had serum glucose levels similar to that of PBS-inoculated control mice. However, all mice infected with CB4-V developed severe hypoglycemia (serum glucose 30 mg/dl $\pm$ 2 1 as compared to 131 $\pm$ 14 for PBS-injected controls) within 4 days PI. Fifty percent of these animals became moribund and were euthanized within 7 days PI. The remaining animals exhibited a prolonged hypoglycemia. Serum glucose levels of these mice were more than 3 standard deviations below normal for 5 weeks PI and returned to normal at 8 weeks PI. During the acute phase which lasted 2 weeks, infected mice had distended abdomens and appeared huddled, inactive and weighed 25–30% less than control animals. To detect additional abnormalities in glucose metabolism after infection with CB4-V, glucose tolerance tests were performed 7 days PI. Infected mice cleared the glucose load efficiently (data not shown). Thus, the hypoglycemic animals did not show abnormal glucose tolerance.

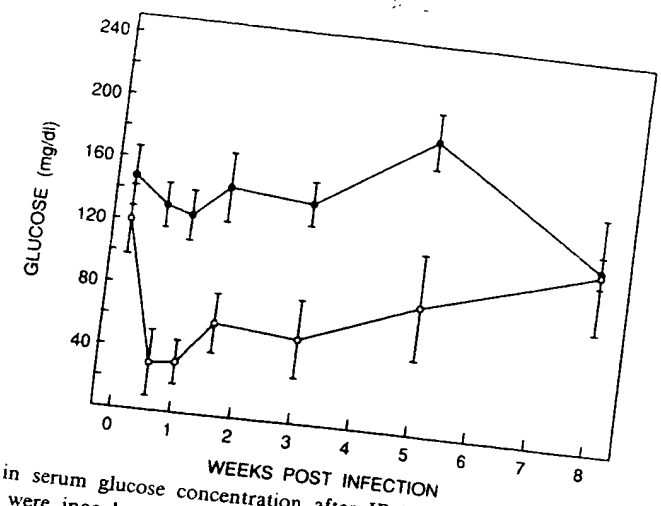


Fig. 1. Reduction in serum glucose concentration after IP inoculation with CB4-V. Groups of 30 4–6-week-old mice were inoculated with $10^{4.3}$ $TCID_{50}$ of CB4-V (○——○) and serum glucose concentrations were monitored for 8 weeks post infection. Fifty percent of these animals were moribund within 7 days PI. Groups of 5–10 4–6-week-old mice were inoculated with PBS (●——●) and serum glucose concentrations were again monitored for 8 weeks. No deaths occurred in this group.

The intensity and duration of the hypoglycemia observed in CB4-V-infected SJL mice could be attenuated by decreasing the virus inoculum. Infection with $10^{2.7}$ $TCID_{50}$ of CB4-V resulted in a less severe and prolonged hypoglycemia (Table 1). The mortality rate for these animals was 0%.

TABLE 1

Susceptibility of various mouse strains to CB4-V

| Mouse strain | H-2 haplotype | | | Mortality rate [a] (%) | Duration of hypoglycemia [b] (weeks) | Severity of symptoms at 7–14 days PI [c] |
|---|---|---|---|---|---|---|
| | K | I | D | | | |
| B10.T(6R) | q | q | d | 100 | 2 | + + + + [d] |
| B10.Q | q | q | q | 43–69 | 6–7 | + + + |
| B10.AQR | q | k | d | 45–60 | 6–7 | + + + |
| B10.AKM | k | k | q | 0 | 3–4 | + |
| B10.S(12R) | s | s | s | 0 | 3–4 | + |
| SJL | s | s | s | 0 | 2–3 | + |

[a,b,c] Summary of results obtained from 3 independent experiments. The shortened duration of hypoglycemia observed in infected B10.T(6R) mice reflects the fact that these animals were moribund within a 2-week period.

[d] Symptoms were monitored daily and graded from mild (+) to extremely severe (+ + + +). +: huddled, active; + + +: huddled, inactive, weight loss; + + + +: huddled, inactive, shivering, weight loss (these animals were defined as moribund).

*CB4-V causes severe acinar pancreatitis in SJL mice*

To correlate histological changes in the pancreas with CB4-V-induced hypoglycemia, tissue from animals infected with $10^{4.3}$ $TCID_{50}$ of virus, was harvested at various times PI and processed for staining by hematoxylin and eosin (Fig. 2A–C). At 4 days PI, degeneration of the exocrine pancreas was observed in SJL mice as a generalized degranulation of the acinar cells and partial loss of the number of exocrine secretory units. Upon histological examination, the interlobular ducts and interstitial connective tissue remained intact. At this time point, the islets of

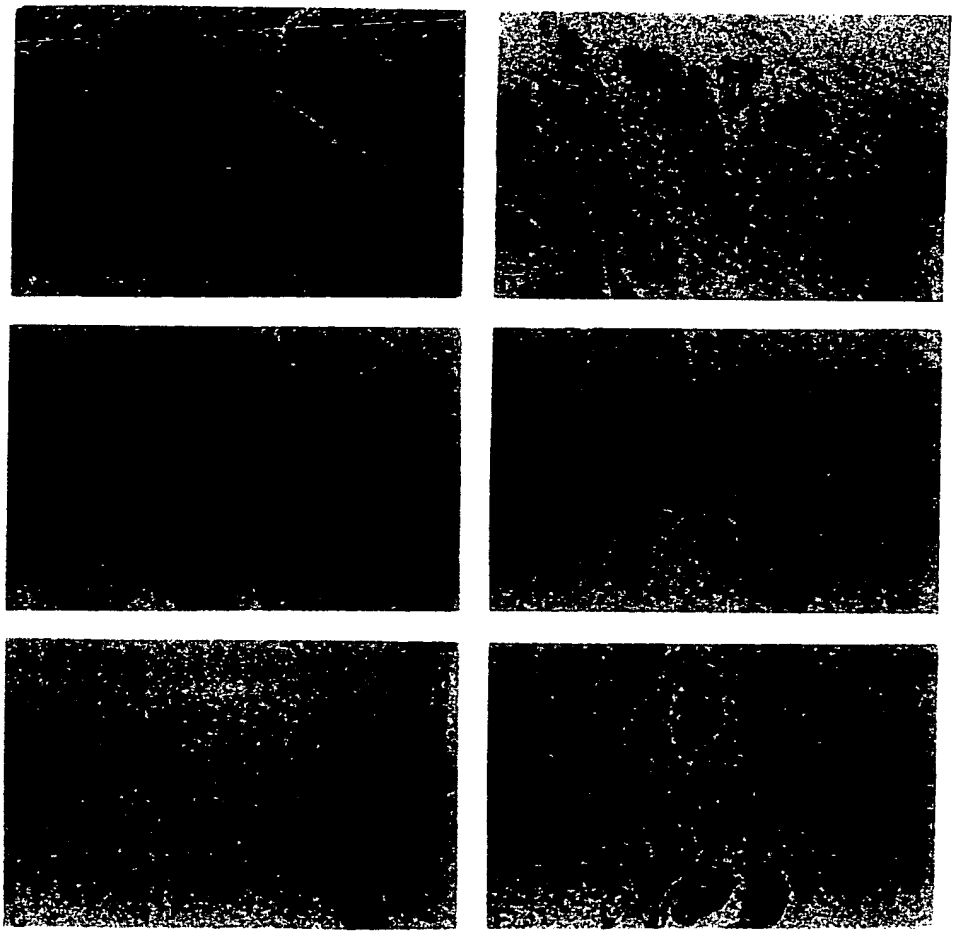

Fig. 2. Histopathology of pancreatic tissue from SJL and B10 mice. SJL and B10 mice were infected with $10^{4.3}$ and $10^{2.7}$ $TCID_{50}$ of CB4-V, respectively. At various times PI, pancreatic tissue was stained with hematoxylin and eosin. (A), PBS-injected control SJL; (B), SJL at 4 days PI; (C), SJL at 8 weeks PI; (D), B10.T(6R) at 4 days PI; (E), B10.S(12R) at 4 days PI; (F) B10.S(12R) at 8 weeks PI. All panels represent equal magnification. Arrows indicate islets of Langerhans.

Langerhans did not show any changes at the light-microscopic level. The degeneration of the exocrine secretory units was progressive so that, by 8 weeks PI, the exocrine pancreas consisted of interlobular ducts and interstitial connective tissue with little or no acinar cells. Still, the islets of Langerhans appeared unchanged and were observed within the remnants of the exocrine pancreas.

*Severity and duration of CB4-V-induced hypoglycemia is influenced by the MHC genes*

To determine whether susceptibility to CB4-V was influenced by genes within the MHC, five H-2 congenic mouse strains were injected IP with $10^{2.7}$ $TCID_{50}$ of CB4-V, CB4-P or 0.2 ml PBS. Serum glucose concentrations were then monitored at various times PI. A low dosage of virus was chosen since, at this concentration, acinar pancreatitis with concurrent hypoglycemia was induced in SJL mice while the

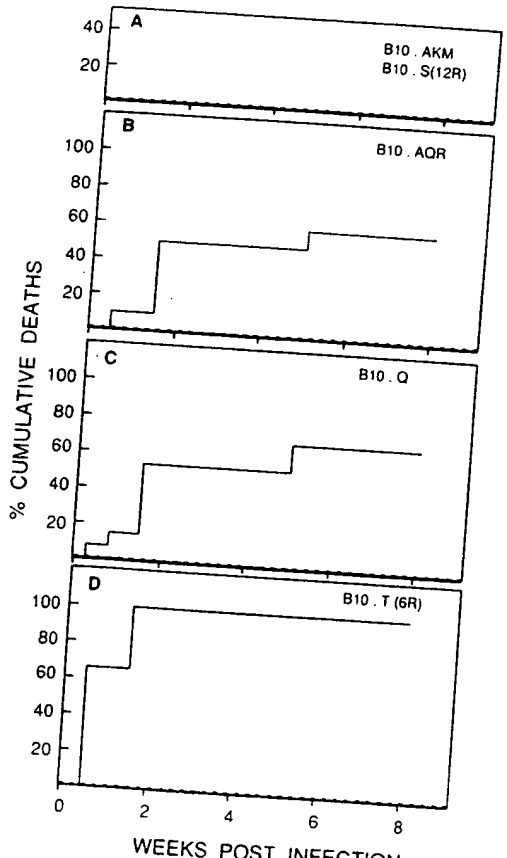


Fig. 3. Cumulative deaths of B10 H-2 congenic mice infected with a low dose, $10^{2.7}$ $TCID_{50}$, of either CB4-V (———) or CB4-P (- - - - -) virus. (A), strains B10.AKM, B10.S(12R); (B), B10.AQR; (C), B10.Q; (D), B10.T(6R).

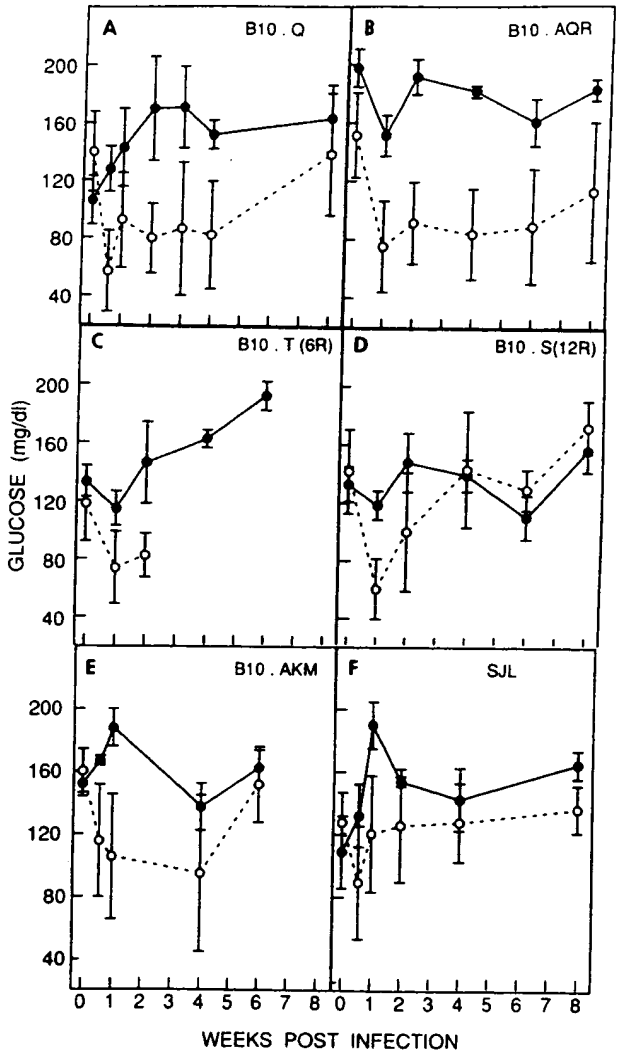


Fig. 4. Serum glucose levels of various mouse strains after IP inoculation with CB4-V (○------○) or with PBS (●———●). Groups of 10–15 4–6-week-old male mice were inoculated with $10^{2.7}$ $TCID_{50}$ of CB4-V and serum glucose concentrations were monitored for 6–8 weeks PI. Infected B10.T(6R) mice were moribund within a 2-week interval. Groups of 5–10 4–6-week-old male mice were inoculated with 0.2 ml of PBS and serum glucose concentrations were monitored for 6–8 weeks. (A), B10.Q; (B), B10.AQR; (C), B10.T(6R); (D), B10.S(12R); (E), B10.AKM; (F), SJL.

mortality rate was 0%. Groups of 5 animals were inoculated with CB4-P or PBS while groups of 10–15 animals were inoculated with CB4-V. These experiments were repeated a total of 3 times and representative results are shown in Figs. 3 and 4. Regardless of strain, mice that were inoculated with either PBS or CB4-P, lived during the follow-up eight weeks PI. In contrast, the B10 H-2 congenic mouse

strains injected with CB4-V fell into either of 2 groups. One group consisting of B10.AQR, B10.Q and B10.T(6R) exhibited 60–100% mortality rates. The second group, consisting of B10.AKM and B10.S(12R), had 0% mortality rates. The severity and duration of hypoglycemia in B10.AKM and B10.S(12R) mice was less than that observed in B10.AQR, B10.Q and B10.T(6R) mice (Fig. 4, Table 1). Mice in the latter group displayed more pronounced shivering, huddling, weight loss and general inactivity than mice in the former group (Table 1). To determine whether non-H-2 genes affected the mortality rate when a low dose of virus was used, CB4-V infected B10.S(12R) mice were compared to CB4-V infected SJL mice which have the same H-2 haplotype i.e. H-$2^s$. Infected SJL mice displayed symptoms signs to those of infected B10.S(12R) mice and the mortality rate was 0%. Since B10.AQR, B10.Q and B10.T(6R) share the K region of H-2, the severity and duration of hypoglycemia observed in CB4-V-infected B10 H-2 congenic mice was associated with H-$2K^q$.

Of the 5 B10 H-2 congenic mouse strains studied, the most susceptible [B10.T(6R)] and one of the more resistant [B10.S(12R)] strains were chosen for histological examination. Pancreases were harvested from CB4-V-infected 4–6-week-old male mice at different times PI and processed for staining by hematoxylin and eosin (Fig. 2D–F). At 4 days PI, as observed in SJL mice that were infected with a higher titer of virus, in both B10 H-2 congenic mouse strains (Fig. 2D, E), there was degranulation of the acinar cells and loss of the exocrine secretory units. Again, the islets of Langerhans did not show any alterations at the light microscopic level. Unlike the SJL mice, the B10 mice exhibited lymphocytic infiltration into the areas of the exocrine cells. In B10.T(6R) mice, a generalized lymphocytic infiltration that appeared to correlate with destruction of the exocrine pancreas was observed (Fig. 2D). These mice were moribund within 2 weeks PI. For the less susceptible strain, B10.S(12R), focal lymphocytic infiltration was observed (Fig. 2E). This infiltration again correlated with destruction of the exocrine pancreas since tissue free from such infiltrates appeared normal. By 8 weeks PI in the B10.S(12R) mice, foci of lymphocytic infiltration were no longer apparent but regions of degenerative exocrine tissue persisted (Fig. 2F). Unlike CB4-V-infected mice, pancreases harvested from B10.T(6R) and B10.S(12R) congenic mice infected with $10^{2.7}$ $TCID_{50}$ of the prototypical virus (CB4-P) showed only minor changes in the exocrine tissue, such as shrunken acinar cells with smaller nuclei; lymphocytic infiltrates were not observed (data not shown).

*Isolation of virus from organs of infected mice*

To determine whether CB4 virus replicated differentially in B10.T(6R) and B10.S(12R) mice, various organs were harvested from mice infected with $10^{2.7}$ $TCID_{50}$ of virus at 4 days PI when viral titers were maximal (data not shown). In each experiment, organs from 3–4 mice were pooled and homogenates were titered for the presence of infectious virus (Table 2). Both CB4-V and CB4-P replicated well in B10.T(6R) and B10.S(12R) mice. Viral titers were higher in the pancreas and spleen than in the kidney or heart. In both mouse strains, CB4-V replicated

TABLE 2

Virus titers obtained from various organs of infected B10 H-2 congenic mice

| Virus | Mouse strain | Virus titer [a] ($-\log_{10}$ $TCID_{50}$/g tissue) | | | |
|---|---|---|---|---|---|
| | | Heart | Kidney | Spleen | Pancreas |
| CB4-P | B10.T(6R) | 3.2 | 3.9 | 7.2 | 6.9 |
| | B10.S(12R) | 2.8 | 4.0 | 5.4 | 7.1 |
| CB4-V | B10.T(6R) | 3.0 | ND [b] | 4.7 | 6.0 |
| | B10.S(12R) | 3.7 | 3.5 | 3.9 | 5.2 |

[a] Virus titers from 3 independent experiments were determined and were reproducible within a 10-fold range. The microtiter $TCID_{50}$ assay used in these experiments is accurate to $10^{0.5}$ (Minor, 1985). Representative results of a single experiment are shown in this Table.

[b] Not detected.

maximally in pancreas, which probably reflects the fact that this virus was derived by passaging pancreatic homogenates in vivo.

## Discussion

The pathogenesis of Coxsackie B virus infections is complex and has been shown to be influenced by both host factors such as age, sex and strain (Khatib et al., 1980) and by intrinsic factors such as virus type and passage history (Toniolo et al., 1982). These experiments addressed whether or not infection by CB4 viruses (CB4-P and CB4-V) is dependent on MHC haplotype.

A pancreatropic variant, CB4-V, was generated by passaging virus in mice via pancreatic homogenates. In SJL mice, this virus caused a prolonged and severe hypoglycemia with concurrent pancreatis as is shown by degeneration of the acinar cells of the exocrine pancreas. Lymphocytic infiltration of pancreatic tissue was not observed. Infection of various B10 H-2 congenic mouse strains revealed that the development of hypoglycemia with accompanying pancreatitis was MHC-independent. However, disease outcome, as monitored by the severity and duration of hypoglycemia and by mortality rate, was associated with the H-2 haplotype. Three strains of mice, B10.AQR, B10.Q and B10.T(6R) had high mortality rates (43–100%) while mice of strains B10.AKM and B10.S(12R) were not killed by the virus. Thus, in B10 mice, the severity of disease induced by CB4-V correlated with the H-$2K^q$ locus. Although CB4-P replicated well in both B10.T(6R) and B10.S(12R), these mice were normoglycemic and did not appear ill during the follow-up eight weeks post-infection. This suggested that like CB4-V, infection of B10 H-2 congenic mice with CB4-P was MHC-independent.

Since the K locus gene product can act to restrict the response of cytotoxic T lymphocytes (CTL) to viral antigens, several possibilities can be proposed to explain the correlation of severity of CB4-V infection with $K^q$. One possibility is that $K^q$ acts as a major restriction element for the antigens of CB4-V, resulting in an increased CTL response in the B10.AQR, B10.Q and B10.T(6R) mouse strains as

compared to the B10.AKM and B10.S(12R) strains. Thus, pancreatic damage would be due primarily to immune-mediated destruction of infected acinar cells by CTL. As an example, susceptibility to lymphocytic choriomeningitis virus (LCMV) is linked to H-2D$^q$ (Zinkernagel et al., 1985). LCMV can persist in pancreatic $\beta$ cells of adult mice and is associated with aberrations in blood glucose concentrations (Tishon and Oldstone, 1987). Furthermore, LCMV-induced disease is the result of a T cell-mediated pathophysiological mechanism (Zinkernagel et al., 1985). A second possibility is that K$^q$ does not function as a restriction element for CB4-V. This lack of recognition by CTL of viral-infected cells should result in higher titers of virus in infected tissue due to increased viral replication and spread. Pancreatic damage in this case would be due primarily to viral-mediated destruction. Khatib et al. (1987) have suggested that myocardial damage in the acute phase of CB3 infection is caused by direct virus cytopathogenicity rather than by host immune response. We examined whether CB4 virus replicated differentially in a susceptible [B10.T(6R)] and a resistant [B10.S(12R)] strain of mice (Table 2). Both CB4-V and CB4-P replicated to high titers in the pancreas of both strains of mice. However, CB4-V induced a severe hypoglycemia in B10.T(6R) mice, which succumbed to viral infection. CB4-V-infected B10.S(12R) mice exhibited a much milder hypoglycemia and did not succumb to viral infection. Since preferential replication of CB4-V in the most susceptible mouse strain, B10.T(6R), was not observed, this argues against K$^q$ influencing viral replication and destruction. An extension of this argument would be that direct viral destruction is not the major mechanism of pancreatic injury. This is also supported by the fact that CB4-P replicated to high titers in the pancreas of both B10.T(6R) and B10.S(12R) mice yet histological examination revealed no destruction of pancreatic tissue. Histological examination of pancreatic tissue from CB4-V infected B10 H-2 congenic mice revealed an association between acute destruction of the exocrine pancreas and lymphocytic infiltration. In the resistant B10.S(12R) mice, focal infiltrates were observed in the exocrine tissue. However, a generalized infiltration was observed in the exocrine tissue of the susceptible B10.T(6R) mice. This generalized lymphocytic infiltration may correlate with immune-mediated destruction of pancreatic tissue in B10 mice but not in SJL mice. To determine whether pancreatic damage in CB4-V infected mice is a result of immune-mediated destruction, we are currently testing whether K$^q$ acts as a restriction element for viral antigens by measuring the CTL response of the B10 H-2 congenic mice to CB4-V. In addition to examining the role of host genes in the pathogenesis of CB4 virus infections, hybrid viruses have been constructed from cDNA clones of both CB4-V and CB4-P to localize the gene(s) involved in disease induction (Ramsingh et al., manuscript in preparation).

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# 1: S39291. Coxsackievirus B4...[gi:250908]

LOCUS S39291 3298 bp RNA linear VRL 03-AUG-1999
DEFINITION Coxsackievirus B4 VP4 gene, partial sequence; VP2 (VP2) gene, partial cds; VP3 gene, partial sequence; and VP1 (VP1) gene, partial cds.
ACCESSION S39291
VERSION S39291.1 GI:250908
KEYWORDS .
SOURCE Human coxsackievirus B4
ORGANISM Human coxsackievirus B4
Viruses; ssRNA positive-strand viruses, no DNA stage; Picornaviridae; Enterovirus.
REFERENCE 1 (bases 1 to 3298)
AUTHORS Ramsingh,A., Araki,H., Bryant,S. and Hixson,A.
TITLE Identification of candidate sequences that determine virulence in Coxsackievirus B4
JOURNAL Virus Res. 23 (3), 281-292 (1992)
PUBMED 1320798
REMARK GenBank staff at the National Library of Medicine created this entry [NCBI gibbsq 108105] from the original journal article.

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